



Deciphering the early events leading to an adaptive immune response during urinary tract infection

Gabriela Mora Bau

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Université Pierre et Marie Curie

École doctorale Physiologie et Physiopathologie (ED 394)

Immunobiologie des Cellules Dendritiques (Unité Mixte Pasteur/Inserm U818)

Définir le début des événements conduisant à une réponse immunitaire adaptative lors de l'infection urinaire

Par Gabriela Mora Bau

Thèse de doctorat de Immunologie

Dirigée par Molly A. Ingersoll, PhD

Présentée et soutenue publiquement le 30 Septembre 2015

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Résumé

L'infection des voies urinaires est l'une des infections bactériennes les plus courantes avec des coûts de soins de santé très élevés. On estime que 50% des femmes connaîtront une infection urinaire au cours de leur vie, ceci de manière récurrente chez la moitié d'entre elles. Le développement de thérapies efficaces a été limité par le manque de connaissance concernant la mise en place de la réponse immune adaptative lors de cette infection. Dans cette étude, nous avons démontré qu'une réponse adaptative est générée lors de l'infection urinaire, cependant celle-ci n'a pas d'action protectrice. Afin de comprendre les mécanismes aboutissant à ce phénomène, nous avons cherché à caractériser les cellules immunitaires présentes dans la vessie. Des tests d'absorption bactérienne ont montré que ces macrophages phagocytent la majorité des bactéries au début de l'infection. Pour évaluer l'influence de ces cellules sur la mise en place de la réponse immune adaptative, nous avons déplété les macrophages et évalué la clairance bactérienne lors d'une deuxième infection. En comparaison avec les animaux non traités, les souris déplétées présentaient une réduction de la charge bactérienne consécutive lors de la seconde infection, cette clairance dépendant de la réponse immune adaptative. Pour comprendre ce mécanisme d'inhibition par les macrophages, nous avons évalué le microenvironnement vésical et la phagocytose au début de l'infection chez les souris déplétées, et chez les souris non traitées. Bien que nous n'ayons pas observé de différences dans la production de cytokines, l'absorption bactérienne par les cellules dendritiques s'avère deux fois plus importante chez les animaux déplétés. Ces données suggèrent que l'absorption bactérienne par les macrophages tissulaires est néfaste pour la mise en place de la réponse adaptative, ouvrant de nouvelles options thérapeutiques. Nous avons également évalué le rôle des lymphocytes T dans ce processus en déplétant ces cellules au cours de l'infection primaire ou avant la deuxième infection. Ainsi, nous avons observé que les lymphocytes T sont nécessaires dans la réponse adaptative, mais ne sont cependant pas indispensables à la clairance bactérienne lors d'une réinfection. De plus, l'infection des souris *Batf3^{-/-}*, déplétées en cellules dendritiques spécialisées dans la présentation croisée, a montré que ces souris contrôlent une seconde infection aussi bien que les souris contrôle. Ces résultats suggèrent que la présence lymphocytes T $CD8^+$ n'est pas nécessaire pour lutter contre l'infection urinaire. Notre étude révèle un mécanisme par lequel le système immunitaire est compromis lors de l'infection urinaire, offrant un point de départ intéressant pour une recherche plus approfondie sur le rôle du système immunitaire adaptatif dans ce contexte, élément fondamental dans le développement de nouvelles thérapies.

Abstract

Urinary tract infection (UTI) is one of the most common bacterial infections with exorbitant health care costs. It is estimated that 50% of women will experience a UTI during their lifetime and approximately half will suffer recurrent infections. Infected women are treated with antibiotics, however, antibiotic resistance is increasing, raising the need for new therapeutic options. Development of efficient therapies has been impeded by the lack of knowledge of events leading to adaptive immunity. In this study, we demonstrated that an adaptive immune response is generated during UTI, however this response does not confer protective immunity. To begin to understand why the response induced during UTI was not effective, we delineated the immune cell compartment of the bladder and identified macrophages as the most populous immune cell. We evaluated bacterial acquisition in the bladder observing that macrophages phagocytize the majority of the bacteria early in infection. To evaluate the impact of macrophages on the generation of adaptive immunity, we depleted bladder resident macrophages and evaluated bacterial clearance during a challenge infection. Interestingly, mice depleted of resident macrophages, prior to primary infection, exhibited a nearly 2-log reduction in bacterial burden following secondary challenge compared to untreated animals. This improvement in clearance was dependent on the adaptive immune system. To shed light on the mechanism of macrophage inhibition, we evaluated the bladder microenvironment and bacterial acquisition early in infection in macrophage-depleted and control-treated mice. While we did not observe differences in the cytokine microenvironment, bacterial uptake by dendritic cells was increased nearly 2-fold in macrophage-depleted animals. These data suggest that bacterial uptake by tissue macrophages negatively impacts the development of adaptive immunity, revealing a novel target for enhancing host responses to bacterial infection of the bladder. We also evaluated the role of T cells during UTI by depleting these cells during the course of the infection or just prior to challenge infection. We observed that T cells were necessary to mount an adaptive immune response to UTI, however, they were dispensable for bacterial killing during challenge infection. Additionally, infection of *Batf3*^{-/-} mice, lacking cross-presenting dendritic cells, suggested that CD8⁺ T cells are dispensable for the response against UTI as these mice cleared a challenge infection as well as wildtype mice. Our study has revealed a mechanism by which the immune system is compromised during UTI, providing an interesting start point for further investigation of the role of the adaptive immune system during UTI, which will be fundamental for the development of new therapies to efficiently treat infection.

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Abbreviations

APC	Antigen presenting cell
BCAM	Basal cell adhesion molecule
BCG	Bacillus Calmette-Guerin
cAMP	Cyclic AMP
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CDC42	Cell division control 42
CFU	Colony forming unit
CNF1	Cytotoxic necrotizing factor 1
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DT	Diphtheria toxin
ELISA	Enzyme-linked immunosorbent assay
ExPEC	Extraintestinal pathogenic <i>Escherichia coli</i>
GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
HlyA	Alpha-hemolysin
IBC	Intracellular bacterial community
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MMP9	Matrix metalloproteinase 9
MOI	Multiplicity of infection
NK	Natural killer
OVA	Ovalbumin
P.I.	Post infection
QIR	Quiescent intracellular reservoir
RFP	Red fluorescent protein
rRNA	Ribosomal RNA
Th	T helper

THP	Tamm-Horsfall protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPEC	Uropathogenic <i>Escherichia Coli</i>
UTI	Urinary tract infection
WT	Wild type

Chapter 1: Introduction

The bladder mucosa

The bladder is a unique mucosal surface composed of a transitional epithelium containing three to six layers which are organized as follows: facing the luminal surface of the bladder are highly differentiated, large and multinuclear superficial cells (referred to as umbrella or facet cells), one or more intermediate cell layers, and a basal cell layer on top of the lamina propria (Figure 1) (Apodaca, 2004; Ingersoll and Albert, 2013). The capacity of the bladder to regenerate after damage was described many years ago (Hicks, 1975), however the presence of stem cells in the bladder was first proposed only very recently in 2008 (Kurzrock et al., 2008). In 2011, it was demonstrated that basal cells of the uroepithelium include stem cells. These stem cells are able to self-renew and differentiate into all the cell types composing the uroepithelium (Shin et al., 2011). Like in other tissues, resident immune cells are present in the bladder mucosa. MHC II⁺ cells were detected by histology in humans, pigs, and mice (Gardiner et al., 1986; Hart and Fabre, 1981a; Hjelm et al., 1982). Additionally, CD11c⁺ and F4/80⁺ cells (Engel et al., 2008; Schilling et al., 2003), as well as T cells (Christmas, 1994) have been identified in steady state and infected mouse bladders.

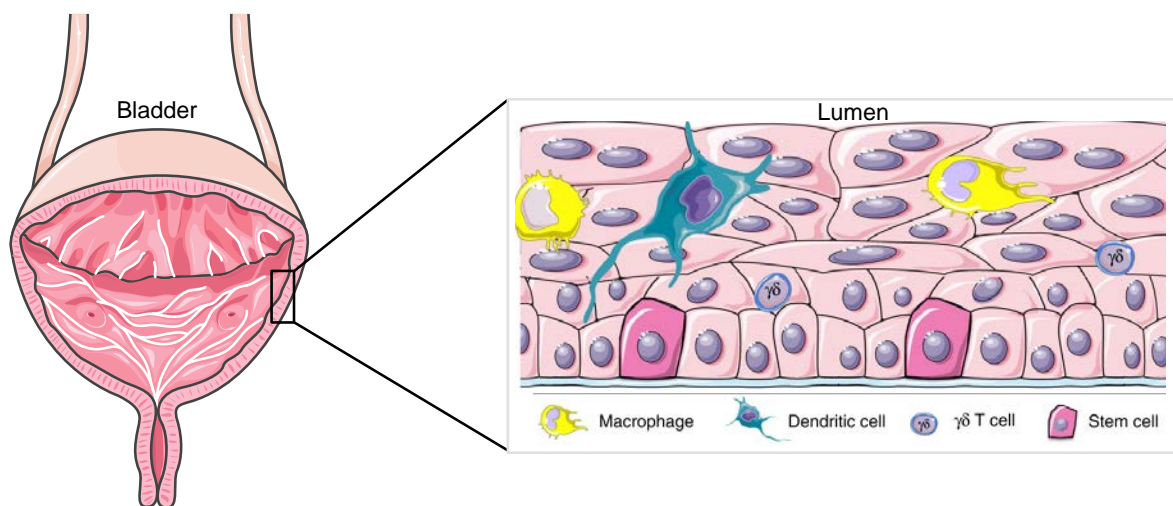


Figure 1: Schematic representation of the bladder uroepithelium. The bladder uroepithelium is composed of 3–6 uroepithelial cell layers. Multinuclear umbrella cells face the lumen. Intermediate and basal layers are directly underneath. Stem cells, in dark pink, are found in the basal layer, on top of the lamina propria (delineated by the pale blue line). The bladder contains resident $\gamma\delta$ T cells and phagocytes such as macrophages and dendritic cells. Figure modified from Ingersoll and Albert, 2013.

The apical side of umbrella cells is covered with numerous rigid-looking plaques (2 dimensional crystals of hexagonally packed 16-nm protein particles) (Kachar et al., 1999; Wu et al., 2009) (Figure 2). These plaques are composed of uroplakins, which are integral membrane proteins (Apodaca, 2004). Four uroplakin proteins have been identified: UPIa, UPIb, UPII, and UPIIIa. The plaques are formed when UPII and UPIa dimerize, as well as UPIII and UPIb; then, the two heterodimers bind to form a tetramer. Finally, 6 tetramers bind together to form a plaque (Hu et al., 2005) (Figure 2). These uroepithelial plaques are in part responsible for the barrier function of the uroepithelium as they help to make the bladder impermeable (Negrete et al., 1996). Additionally, the plaques stabilize the apical surface, and prevent uroepithelial rupture during bladder distension (Staehelin et al., 1972). Moreover, they also have a role during infection, discussed later.

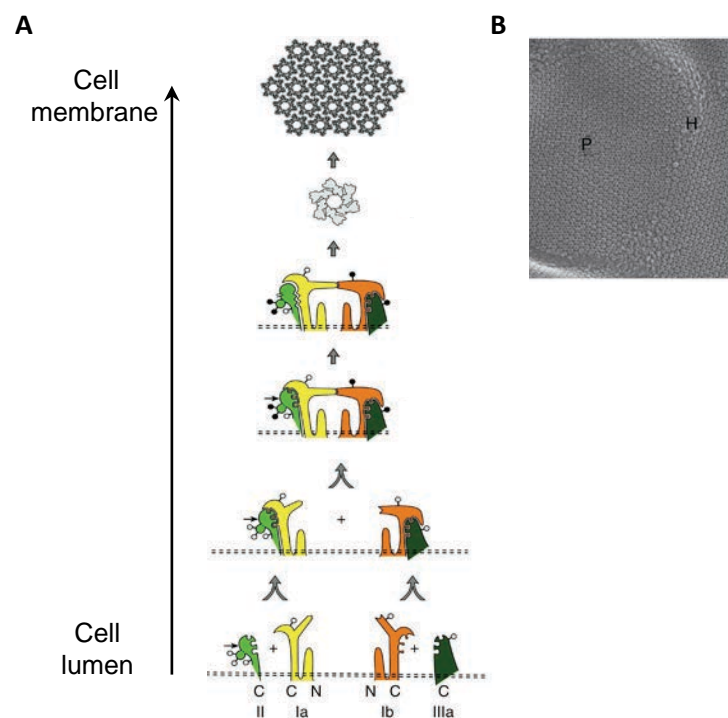


Figure 2: Urothelial plaque formation. (A) A model depicting the assembly of the four major uroplakins (UPIa, Ib, II, and IIIa) into 2D crystals, as described in the text. (B) Quick-freeze deep-etch image of the apical surface of a mouse umbrella cell showing urothelial plaques (P) containing hexagonal arrays of 16-nm particles interconnected by particle-free hinge (H) areas. Figure and legend modified from Wu et al., 2009.

The bladder has been considered a sterile mucosa, lacking colonizing microflora (Zasloff, 2007) however, evidence suggests that similar to other organs, like the gut or skin, commensal bacteria reside in the bladder (Anderson et al., 2004b; Siddiqui et al., 2011). The bladder was thought to be sterile for so long due the inability to detect bacteria in urine samples. However, in 2004, viable but nonculturable bacteria were detected in mouse and human urine samples (Anderson et al., 2004b). Different bacteria have been found in the urine from healthy women through the study of bacterial 16S rRNA sequences, with *Lactobacillus* being the most prominent in the urine microbiota (Siddiqui et al., 2011). Additionally, bacteria genera found in vaginal microbiota were also detected in urine samples from healthy women (Ling et al., 2010; Siddiqui et al., 2011). Bacteria commonly considered as difficult to culture (meaning that they are not generally detectable by conventional culture methods), such as *Aerococcus urinae* and the genus *Ureaplasma*, were also detected in this study (Siddiqui et al., 2011).

In general, the bladder is not as well studied as other mucosal surfaces. This mucosa is the most impenetrable organ in the body and has the important function of protecting the body from toxins accumulated in the urine (Negrete et al., 1996). While the bladder certainly harbors a microbiota, which needs to be more fully investigated, it is also susceptible to different uropathogens, which cause urinary tract infection (UTI), one of the most common infections in the world (Flores-Mireles et al., 2015; Foxman, 2010). Given the importance of this organ, and our relative lack of knowledge of its biology, it is both a relevant and interesting topic of study.

Urinary tract infection

UTI is one of the most common bacterial infections, impacting more than 150 million people annually and resulting in significant health care costs and morbidity (Foxman, 2010; Stamm and Norrby, 2001). In the United States alone, the cost of health care and time missed from work reaches 3.5 billion dollars per year (Foxman, 2014). Women are at greater risk for UTI than men and it is estimated that one out of two women will experience a UTI during their lifetime, while nearly half of these individuals will experience one or more recurrent

infections (Foxman, 2002). Risk factors for UTI include gender, prior UTI, sexual activity, spermicide use, vaginal infection, diabetes, and catheterization, among others (Hooton, 2000).

UTI can be classified as uncomplicated or complicated. Uncomplicated UTI generally affects individuals who are otherwise considered healthy and do not present any structural abnormalities in their urinary tract. Uncomplicated UTI can affect the lower urinary tract (lower UTI or cystitis) or the upper urinary tract (upper UTI or pyelonephritis). Lower UTI is characterized by symptoms such as frequent urination, urgency, dysuria, and abdominal discomfort. If these infections remain untreated, they can progress to upper UTI, which are associated with additional symptoms including nausea, vomiting, fever, and flank pain. Notably, upper UTI can eventually progress to bacteremia (Hannan et al., 2012; Hooton, 2012; Nielubowicz and Mobley, 2010). Contrary to uncomplicated UTI, complicated UTI are associated with individuals who present anatomical or functional abnormalities, or who are not completely healthy and are suffering from other illness or immunosuppression, are undergoing long-term catheterization, or have received renal transplantation (Flores-Mireles et al., 2015; Lichtenberger and Hooton, 2008). Complicated UTI are a significant cause of bacteremia worldwide and are associated with mortality rates of 20–40% among critically ill patients (Chan and Yuen, 2015).

The principal causative agent of uncomplicated and complicated UTI is uropathogenic *Escherichia coli* (UPEC) (Flores-Mireles et al., 2015; Foxman, 2010). In the case of uncomplicated UTI, UPEC causes approximately 75% of all community acquired infections followed by *Klebsiella pneumoniae* and *Staphylococcus saprophyticus*. For complicated UTI, *Enterococcus* species and *Klebsiella pneumonia* follow UPEC in prevalence (Flores-Mireles et al., 2015) (Figure 3). In addition to bacteria, other pathogens can infect the bladder. *Candida albicans* can infect the urinary tract producing the same symptoms as bacterial UTI, however most *Candida albicans* infections are asymptomatic (Fisher et al., 1982; Malani and Kauffman, 2007). In addition to *Candida albicans*, parasitic worms of the *Schistosoma* genus (in particular, *Schistosoma haematobium*) can also infect the urinary tract. These infections are more common in Africa and the Middle East and are associated with an atypical form of bladder cancer (Mostafa et al., 1999; Rosin et al., 1994).

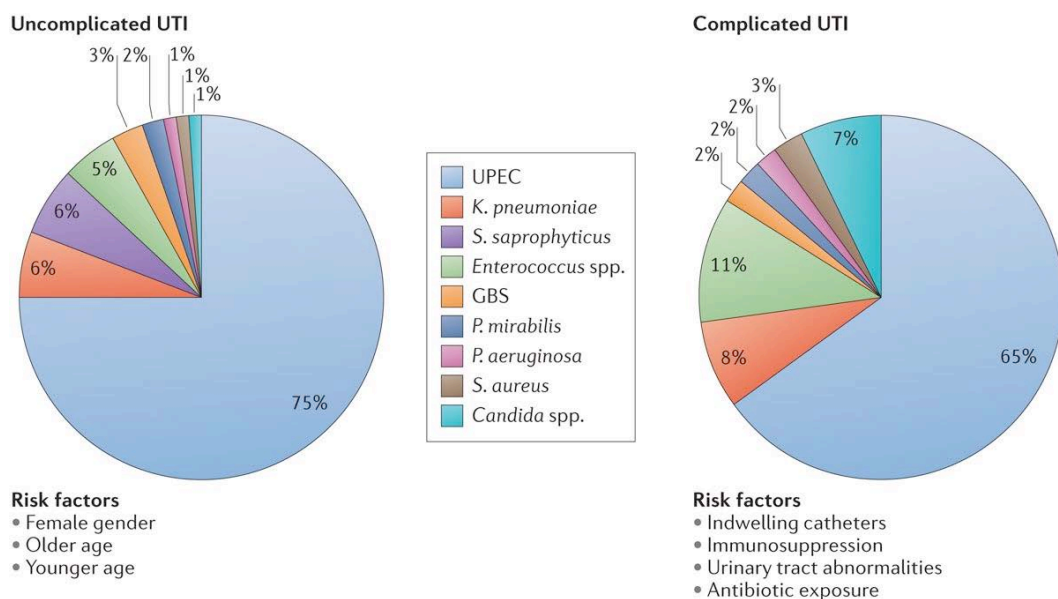


Figure 3: Causative agents of urinary tract infections. Camembert charts illustrate the most common causative agents for both uncomplicated (left) and complicated (right) UTI as well as risk factors associated with both infections. Figure from Flores-Mireles et al., 2015.

UPEC is the main causative agent of UTI

Escherichia coli (*E. coli*) are Gram-negative rod shaped bacteria, which are incredibly diverse and can colonize numerous niches. Most *E. coli* strains are harmless and can even have a beneficial relationship with their host. However, some strains can cause disease and are therefore considered pathogenic (Wiles et al., 2008). These pathogenic strains can be classified as intestinal or extraintestinal *E. coli*. UPEC is classified as an extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000; Wiles et al., 2008). Although UPEC strains are well studied, specific factors differentiating *E. coli* strains unable to infect the urinary tract from UPEC strains (*E. coli* able to infect the urinary tract and cause disease) have not been described. However, it has been observed that UPEC strains share certain sequences among them, such as iron acquisition genes, that are maintained by positive evolutionary pressure (positive selection occurring during bladder colonization) conferring upon UPEC the ability to live in the urinary tract (Chen et al., 2006).

In fact, it has been proposed that instead of a common and unique virulence factor, it is the ability to accumulate and express several virulence genes that defines UPEC strains (Brzuszkiewicz et al., 2006). UPEC possess different virulence factors, which can be classified based on their function as: adherence factors, immune evasion, iron acquisition, toxin, and others (Figure 4). Fimbriae, also known as pili, are complex surface structures that can be found in many Gram-negative bacteria and mediate adherence of bacteria to specific receptors expressed by host cells (Krogfelt, 1991; Proft and Baker, 2009). UPEC express different fimbriae structures including type 1, Dr, P, and S fimbriae (Connell et al., 1996). Type 1 fimbriae are composed of a major structural subunit FimA and the minor subunits FimF, FimG, and FimH. The subunits are assembled by a chaperone/usher pathway consisting of a periplasmic chaperone (FimC) and an integral outer membrane usher protein (FimD) (Figure 5) (Busch and Waksman, 2012; Jones et al., 1995; Waksman and Hultgren, 2009). FimH recognizes mannose-containing host glycoprotein receptors, such as UPIa, which are highly expressed on the apical surface of umbrella cells (Hung et al., 2002). Additionally, $\beta 1$ and $\alpha 3$ integrins, which are located throughout the uroepithelium, also represent key receptors for FimH (Eto et al., 2007). These receptors are essential for UPEC invasion of uroepithelial cells as inhibition of their interaction with UPEC impairs host cell invasion (Eto et al., 2007). Indeed, type 1 fimbriae are absolutely fundamental for UPEC virulence, as they mediate bladder colonization (Connell et al., 1996; Proft and Baker, 2009). UPEC mutants lacking FimH are unable to invade uroepithelial cells (Martinez et al., 2000 ; Wright et al., 2007), leading to severely reduced colonization (Connell et al., 1996; Rosen et al., 2008b). All UPEC strains expression type 1 pili and in addition, P fimbriae are expressed by a subset of UPEC strains and are generally associated with pyelonephritis in humans (Lane and Mobley, 2007). P fimbriae are composed of a major subunit protein called PapA and the minor subunit proteins PapE and PapF followed by PapG at the tip of the fimbria (Kuehn et al., 1992; Lane and Mobley, 2007). It has been shown that type 1 fimbriae work in concert with P fimbriae to promote kidney colonization (Melican et al., 2011).

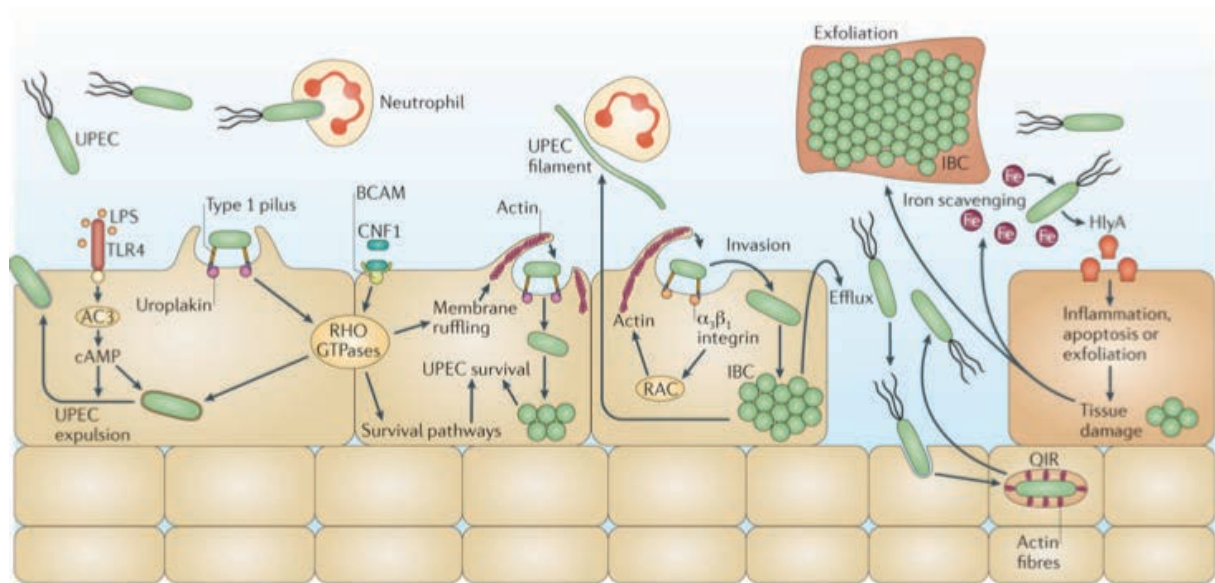


Figure 4: UPEC-host interactions during UTI. In the bladder, UPEC expression of type 1 fimbriae is essential for colonization, invasion, and persistence. FimH binds mannoseylated uroplakins and integrins that coat the surface of umbrella cells. Uroplakin binding by FimH induces actin rearrangement and bacterial internalization via unknown mechanisms. FimH- $\alpha_3\beta_1$ integrin interactions induce actin rearrangement via activation of RHO-family GTPases (such as RAC proteins), resulting in bacterial invasion. Inside the host cell, UPEC can subvert host defenses and resist antibiotic treatment. However, lipopolysaccharide (LPS) released by UPEC is sensed by TLR4, which induces cAMP production via adenylyl cyclase 3 (AC3) activation, resulting in exocytosis of vesicular UPEC across the apical plasma membrane. UPEC subverts this innate defense mechanism by escaping into the cytoplasm, where it then multiplies to form IBCs. Maturation of intracellular bacteria communities (IBCs) causes bacterial dispersal and allows the invasion of other host cells. Alternatively, UPEC can establish quiescent intracellular reservoirs (QIRs) in the underlying transitional cells. In addition, UPEC survives within the harsh bladder environment by secreting several factors that are important for nutrient acquisition. The HlyA promotes host cell lysis and nutrient release through pore formation. The siderophores expressed by UPEC allow the bacterium to scavenge iron and thus promote survival. HlyA also triggers epithelial exfoliation. CNF1 is also important for host cell remodelling and functions by binding to the receptor basal cell adhesion molecule (BCAM) on host cells to induce constitutive activation of the RHO GTPases RAC1, RHOA, and cell division control 42 (CDC42), resulting in actin cytoskeletal rearrangements and membrane ruffling. Activation of RAC1 also induces the host cell anti-apoptotic and pro-survival pathways, preventing apoptosis of colonized epithelial cells. The extracellular survival of UPEC also requires evasion of the innate immune system by the adoption of a filamentous morphology, which renders the bacterium more resistant to neutrophil killing. Figure and legend modified from Flores-Mireles et al., 2015.

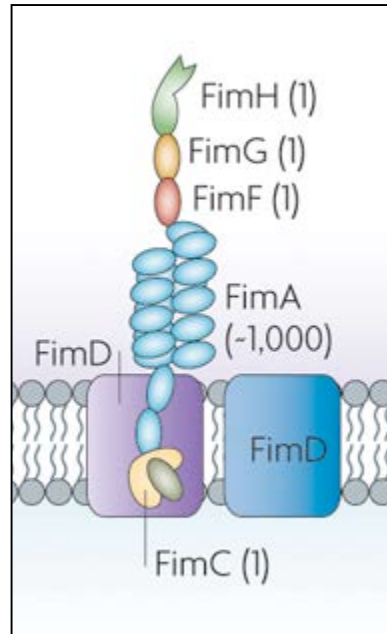


Figure 5: A schematic representation of type 1 fimbriae. Type 1 fimbriae contain FimA, FimF, FimG and FimH (at the tip of the structure) and the fimbriae assembly system FimD and FimC. The numbers indicate the number of copies of each subunit in the fimbria. Modified from Waksman and Hultgren, 2009.

In addition to virulence factors related to adherence, UPEC is able to produce toxins that induce host cells lysis, allowing UPEC to capture nutrients, such as iron, from the cell and to survive in the bladder microenvironment (Dhakal and Mulvey, 2012; Garcia et al., 2013). For example, UPEC secretes alpha-hemolysin (HlyA), which inserts into the umbrella cell membrane and induces pore formation, promoting cell lysis (Dhakal and Mulvey, 2012; Garcia et al., 2013). Additionally, UPEC is able to secrete cytotoxic necrotizing factor 1 (CNF1) that impacts actin remodeling in host cells through the constitutive activation of members of the Rho family of GTP-binding proteins (Garcia et al., 2013). CNF1 binds to the receptor basal cell adhesion molecule (BCAM) and enters into the host cell in endocytic vesicles. It activates Rho GTPases, resulting in cytoskeleton rearrangements and membrane ruffling, which leads to bacteria internalization. Together with RHO GTPase activation, CNF1 activates RAC1 and induces anti-apoptotic pathways in the host cell via activation of the Rac1/PI3K/Akt/IKK/NF- κ B pathway (Miraglia et al., 2007).

UPEC also produces a variety of siderophores during infection (Garcia et al., 2011). Iron availability in the bladder is limited and the role of siderophores is to scavenge iron from the environment to improve pathogen survival (Neilands, 1995). UPEC is able to produce 4

different siderophores including aerobactin, enterobactin, yersiniabactin, and salmochelin (Henderson et al., 2009). One study tested different UPEC siderophore mutants in mixed infections to evaluate their role and/or redundancy during infection (Garcia et al., 2011). Garcia et al. utilized UPEC isogenic mutants lacking individual receptors and performed an *in vivo* series of mixed competitive infections, by instilling mice with different combinations of the mutant strains and evaluating their ability to colonize the bladder. They observed that yersiniabactin and aerobactin play a more critical role than other siderophores in bladder infection (Garcia et al., 2011). During infection, the host immune response can target UPEC bladder colonization mechanisms. However, UPEC deploys different strategies to evade host defenses. For example, neutrophils release the protein lipocalin-2, which recognizes and binds enterobactin, interfering with its ability to supply UPEC with iron (Goetz et al., 2002). To counteract the action of lipocalin-2, UPEC is able to modify enterobactin through glycosylation to form the related siderophore salmochelin and with this modification enterobactin is no longer recognized (Smith, 2007).

The UPEC pathogenic cycle

Once in the urinary tract, UPEC binds and invades umbrella cells, which is critical for colonization of the bladder and establishment of a UTI (Wiles et al., 2008). As detailed above, UPEC strains encode filamentous surface adhesive organelles called type 1 pili, which mediate bacterial attachment to uroepithelial cells (Connell et al., 1996; Langermann et al., 1997). Once UPEC invade the uroepithelial cells, a pathogenic cascade is initiated (Figure 6) (Justice et al., 2004). After invasion, replication starts and a loose collection of bacteria is formed within the cytoplasm of the umbrella cells (Justice et al., 2004). During the first hours of UPEC replication, the bacteria retain their characteristic rod shape and are nonmotile (Justice et al., 2004). The doubling time of the bacteria in this phase of the infection is very fast, ranging between 30-35 minutes. Six to eight hours later, the intracellular bacteria start a maturation process where organized colonies are formed, which possess several biofilm-like properties, such as a polysaccharide matrix (Anderson et al., 2003; Kostakioti et al., 2013). During this phase, bacterial cell length is significantly reduced, generating daughter cells with a coccoid shape. After 10-14 hours of infection, the bacteria occupy almost the entire cytoplasm of the umbrella cell, forming bacterial “pods” or a dense and organized community

with a globular shape known as intracellular bacteria communities (IBCs) (Anderson et al., 2003; Justice et al., 2004). Around 12 hours after infection, bacteria localized on the outer edge of the globular communities start to differentiate into a rod shape, become motile, and dissociate from the IBC. These rod-shaped bacteria are observed within the cytoplasm of the cells as well as fluxing out of the cells to the lumen of the bladder (Justice et al., 2004). It is possible that bacterial fluxing into the lumen of the bladder promotes colonization of additional uroepithelial cells, facilitating the spread of the infection to neighboring cells. Of note, it has also been observed that some of the bacteria growing in biofilm-like formation fail to septate but continue growing, resulting in the formation of filamentous bacteria, which septate at a later timepoint into rod-shaped daughters (Justice et al., 2004). During the fluxing and filamentation process, small groups of bacteria are observed in the cytoplasm of healthy umbrella cells, supporting the idea that fluxing leads to a second round of invasion (Justice et al., 2004). The IBC pathway utilized by UPEC during UTI has also been observed in other Gram-negative uropathogen infections that express type 1 pili, such as *Klebsiella pneumoniae* (Hannan et al., 2012; Rosen et al., 2008a). In the first hours of infection, the bladder goes through an exfoliation process and several umbrella cells are lost in the process. Exfoliated cells containing IBCs and filamentous bacteria have been found in the urine of women with acute cystitis, suggesting that the IBC pathway may occur in humans as well as in mice (Rosen et al., 2007). Additionally, UPEC strains obtained from patients with cystitis are able to go through the IBC cycle in a mouse model of UTI, supporting the idea that this pathway can occur in humans (Garofalo et al., 2007).

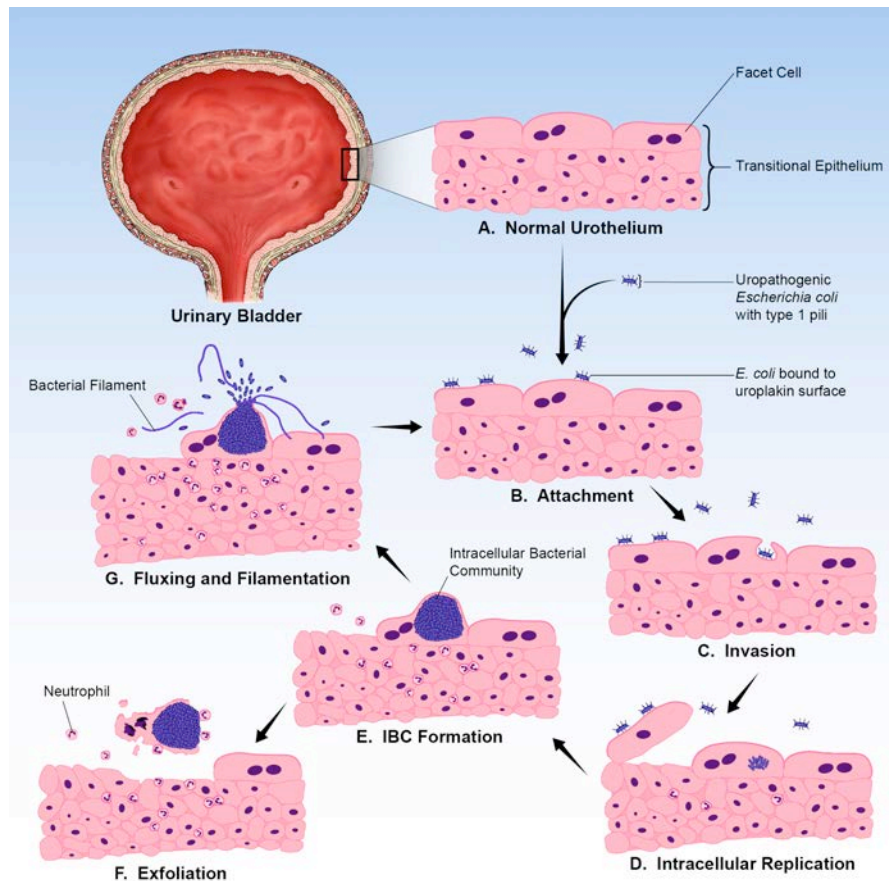


Figure 6: UPEC IBC Pathogenic Pathway Observed in the Murine Cystitis Model. The bladder uroepithelium (A) is a pseudostratified transitional epithelium lined by large facet (umbrella) cells. These cells have an apical asymmetric unit membrane containing uroplakins that help form the impermeable bladder barrier and also serve as receptors for UPEC. Bacteria introduced into the bladder adhere to the bladder surface via type 1 pili (B). Upon attachment, bacteria are able to invade (C) and replicate (D) within the facet cell cytoplasm. UPEC form large biofilm-like IBCs within these cells (E). Ultimately the bacteria flux out of their intracellular niche (G), some adopting a filamentous morphology; they then adhere to other host cells and re-enter the infectious cycle. During this process, infected uroepithelial cells are sloughed into the urine (F) and neutrophils are recruited to the site of infection. Figure and legend from Rosen et al., 2007.

Notably, during infection, UPEC is able to establish intracellular reservoirs, which can persist for months in the bladder tissue. Although not a lot is known about reservoir formation, it has been demonstrated that they are established early during infection (Mulvey et al., 2001; Justice et al., 2004). In a C57Bl/6 mouse model of UTI, animals are able to clear acute infection in a couple of weeks as their urine is sterile and there is no evidence of

inflammatory cells (Justice et al., 2004). However, analysis of bladder tissue revealed the presence of bacteria in umbrella cells arranged in rosette-like clusters, which are defined as bacteria reservoirs. These clusters of bacteria remain quiescent (they do not replicate) over weeks and are sequestered within Lamp1⁺ endosomes inside uroepithelial cells (Mysorekar and Hultgren, 2006). Antibiotics are unable to eradicate UPEC reservoirs in mice (Blango and Mulvey, 2010). Given that reservoirs are not eliminated with antibiotic treatment and that eventually bacteria present in the reservoirs can re-emerge and start a new cycle of infection, it has been proposed that reservoirs contribute to the high rates of UTI recurrence (Barber et al., 2013; Schilling et al., 2002). Supporting this hypothesis, recurrence infections are frequently caused by the same bacterial strain that caused a previous infection in patients (Brauner et al., 1992; Chen et al., 2013b; Ikaheimo et al., 1996). However, there is not enough direct evidence to the date to support the existence of bacteria reservoirs in humans, therefore further studies are needed.

Therapeutic options for UTI

Antibiotics, such as ampicillin, trimethoprim sulfamethoxazole, and ciprofloxacin are most commonly used for the treatment of patients with UTI, however antibiotics do not prevent recurrence (Foxman, 2010). Women suffering from recurrent infections are advised to take continuous low-dose antibiotic prophylaxis or self-initiated treatment (Nickel, 2005). The frequency of sexual intercourse is the main risk factor for recurrent UTI in young women and postcoital antibiotic therapy to prevent UTI episodes can be employed (Kodner and Thomas Gupton, 2010; Nickel, 2005). Importantly, however, antibiotic resistance in UPEC strains is increasing and raises the necessity for new therapeutic options (Flores-Mireles et al., 2015; Foxman, 2010; Hooton et al., 2004). Antibiotic resistance is actually a matter of great concern, as a recently identified a clone of UPEC, which is globally disseminated, has been described to be multidrug resistant and associated with urinary tract and bloodstream infections in both clinical settings and community acquired infections (Petty et al., 2014; Schembri et al., 2015). The existence of this strain, carrying an easily transmissible resistance cassette, further emphasizes the urgency to explore new alternatives to treat UTI.

Many efforts have been made to develop therapies to specifically target uropathogen virulence factors. Along this line, vaccine development has focused on disrupting bacterial adhesion to the urothelium by targeting bacterial pili. While vaccination with the whole pili structure failed to protect against UTI (Goluszko et al., 2005), it has been observed that adhesion-based vaccines are effective at preventing the establishment of UTI by limiting host-pathogen interactions. Notably, vaccination with FimC-FimH chaperone-adhesin complexes protected nonhuman primates and mice against UTI (Asadi Karam et al., 2013; Langermann et al., 2000; Langermann et al., 1997). The effectiveness of these FimC-FimH vaccines was due to their ability to induce an adaptive immune response (particularly, a humoral response) in vaccinated animals (Asadi Karam et al., 2013; Langermann et al., 2000; Langermann et al., 1997). Vaccines to target surface structures, bacterial toxins, and iron acquisition systems, as well as vaccination with whole bacteria, have also been tested in mice and nonhuman primates with diverse effectiveness (Sivick and Mobley, 2010) (Table 1).

In humans, several immunization treatments have been tested to prevent UTI (Grischke and Rutters, 1987; Sivick and Mobley, 2010; Uehling et al., 2001). In 2007, a phase 2 clinical trial showed that women vaccinated with vaginal suppositories containing heat-killed uropathogenic bacteria significantly reduced the recurrence rate of UTI compared with women vaccinated with placebo suppositories (Hopkins et al., 2007). Importantly, these women did not suffer from significant adverse effects during the treatment. However, frequency of sexual intercourse, a significant risk factor for recurrence in young women, was not taken into consideration in this study and, although improbable, it is possible that the group receiving placebo had sexual relations more often than treated women (Hopkins et al., 2007). Moreover, Hopkins et al. evaluated the antibody response during their trial and did not observe significant differences between placebo and vaccine-treated women. Treatment with a daily oral capsule composed of a lyophilized mix of membrane proteins from 18 *E. coli* strains has also been tested. This treatment reportedly triggers several immunological effects *in vitro*, such as NK cell activation and induction of DC maturation, which are able to activate T cells (Schmidhammer et al., 2002; Van Pham et al., 1990)(Wybran et al., 1989). It also induces specific antibodies in mice and humans and reduces the incidence of UTI in patients (Baier et al., 1997; Bauer et al., 2005; Czerwionka-Szaflarska and Pawlowska, 1996; Huber et al., 2000). Unfortunately, the necessity of daily administration can be unreasonable due to complications of toxicity of the treatment. As none of these vaccines has been

overwhelmingly efficacious, they are not available on the market and further studies to increase immunogenicity and/or decrease toxic side effects need to be pursued.

In addition to vaccination, other strategies have also been tested to treat UTI. One of the alternatives includes treating the bladder with protamine sulfate, a highly cationic protein, to induce umbrella cell exfoliation and in this way, eliminate bound and intracellular UPEC (Mysorekar and Hultgren, 2006). Of note, protamine sulfate treatment eliminated bacterial reservoirs in mice (Mysorekar and Hultgren, 2006). However, protamine sulfate treatment generates high levels of discomfort in healthy volunteers (Lilly and Parsons, 1990). As another strategy, it has been shown that when UPEC invades uroepithelial cells, it can localize to CD63⁺ Rab27b⁺ secretory lysosomes (Bishop et al., 2007). Secretory lysosomes lack degradative capacity and can undergo regulated secretion in response to intracellular Ca²⁺ and cyclic AMP (cAMP) flux. *In vitro* experiments have shown that the use of inhibitors of cAMP activity and Ca²⁺ flux inhibited the exit of intracellular UPEC from uroepithelial cells (Bishop et al., 2007). Interestingly, treating UPEC-infected mice with forskolin (a drug that raises intracellular cAMP levels) induces exocytosis of UPEC-containing intracellular vesicles and exposes bacteria to the extracellular environment (Bishop et al., 2007). Once in the extracellular milieu, UPEC is then susceptible to antibiotic action and immune system attack. Finally, orally active small-molecule FimH antagonists have been tested to treat UTI. These FimH antagonists consist of mannoside compounds that block the interaction of FimH with the host uroepithelial cells, preventing UPEC adherence and invasion. Studies using these antagonists in mice have shown promising results, as they are able to reduce bacterial colonization to almost undetectable levels and also decrease chronic cystitis (defined as persistent, high-titer bacteriuria) (Cusumano et al., 2011). Even though major efforts have been made to find an effective way to treat UTI and to prevent recurrence, to date there is no effective treatment to prevent recurrence and more studies and clinical trials are necessary to achieve this goal.

Category/vaccine type	Tested in ^b	Route ^c	Adjuvant ^d	Immune response ^e	Protection ^f	Reference(s)
Surface structures						
O antigen	R	B, SC	ND ^g	H	B	275
OMP fractions	Swiss	IM, O	CFA	C, H	B	155
Adherence						
Dr fimbria	C3H/HeJ	ND	CFA, IFA	H	—	89
Type 1 fimbria	BALB/c	IM, SC	CFA	H	—	187
FimC-H or FimHt	C3H/HeJ	S	CFA, IFA	H	B, K	154
FimHt	BALB/c	IM, IN	CFA, IFA, CpG	H	B	204
FimC-H	P	IM	MF59	H	U	153
Fim peptides	Swiss	IM, SC	CFA, IFA	H	B	256
P fimbria	BALB/c	IM, SC	CFA, IFA	H	K, U	186, 187, 231
PapD-G	P	IP	AP	H	K	217
Pap peptides	BALB/c	IM, SC	CFA, IFA	H	U, K	231
Toxin						
Denatured HlyA	BALB/c	IM	CFA, IFA	H	K	186
Iron acquisition						
Denatured IroN	BALB/c	SC	ND	H	K	223
Native ChuA	CBA/J	IN	CT	C, H	—	5
Native Iha	CBA/J	IN	CT	C, H	—	5
Native IroN	CBA/J	IN	CT	C, H	—	5
Native Hma	CBA/J	IN	CT	C, H	B, K	5
Native IreA	CBA/J	IN	CT	C, H	B, K	5
Native IutA	CBA/J	IN	CT	C, H	B, K	5
Complex						
SolcoUrovac	BALB/c	IP, V	MO	C	B, K	272
SolcoUrovac	C57BL/6	IP, V	MO	C	B, K	272
SolcoUrovac	Swiss	IP	ND	H	ND	146
SolcoUrovac	R	IP	AP	ND	K	145
SolcoUrovac	P	IM, V	MO	H	B	270
SolcoUrovac	H	IM, V	ND	H	Y	91, 112, 114, 224, 266–269
Uro-Vaxom	BALB/c	IP, O	ND	C, H	ND	15, 117, 233
Uro-Vaxom	H	O	ND	H	U, Y	22, 51, 95, 156, 160, 232, 253
Live, live attenuated, or killed <i>E. coli</i> ^a						
L NU14	C57BL/6J	TU	ND	C, H	B	260
L and K CP9	C57BL/6J	IN	ND	H	ND	219
L and K CP923	C57BL/6J	IN	ND	H	ND	219
LA Δ waal	C57BL/6J	B	ND	ND	B	27
K J96	BALB/c	IM, SC	CFA	H	—	187
K P678-54	BALB/c	IM, SC	CFA	H	—	187
K O6	R	V	IFA	N	B, K	273
K 1677	P	V	MO, MDP	H	U	265, 271

^a L, live; LA, live attenuated; K, killed.
^b If tested in mice, the strain is specified. R, rats; P, nonhuman primates; H, humans.
^c B, bladder; IM, intramuscular; IN, intranasal; IP, intraperitoneal; O, oral; SC, subcutaneous; TU, transurethral; V, vaginal.
^d AP, aluminum phosphate; CFA, complete Freund's adjuvant; CpG, CpG oligodeoxynucleotides; CT, cholera toxin; IFA, incomplete Freund's adjuvant; MDP, muramyl dipeptide; MO, mineral oil (for vaginal route only).
^e C, cellular; H, humoral.
^f K, reduction in kidney colonization/histopathology; B, reduction bladder colonization; —, no protection; Y, significant decrease in UTI incidence; U, reduction of UTI as determined by urinalysis.
^g None disclosed.

Table 1: Previously tested vaccines for UPEC-mediated UTI. From Sivick and Mobley, 2010.

The host response during UTI

Defense mechanisms of the urinary tract

The bladder possesses different mechanisms to prevent the colonization of uropathogens. These include urine flow and expression of highly sulfated and anionic glycosaminoglycans

(GAGs), which line the luminal surface of the bladder, acting as antimicrobial adherence factors (Lilly and Parsons, 1990; Sivick and Mobley, 2010). While urine flow may be thought to be an effective mechanism to eliminate bacteria colonizing the urinary tract, it has been shown that upon an increase in the shear force (like the one occurring with urine flow), FimH acts as a force sensor and *E. coli* actually increase their adherence to target cells (Thomas et al., 2002). Fimbriae are dynamic structures and P pili and type 1 fimbriae are highly extensible, which positively affects the lifetime of the bonds between the host receptors and the bacteria (Miller et al., 2006). Type 1 fimbriae can stretched and relaxed repeatedly and at high shear forces, unraveling increases, resulting in longer FimH-mannose interactions (Miller et al., 2006).

Uroepithelial cells also secrete potent antimicrobial agents in response to UPEC (Sobel, 1997). Antimicrobial peptides are small, positively charged peptides that bind and disrupt bacterial membranes, killing the bacteria (Zasloff, 2007). In mammals, the most commonly produced antimicrobial peptides are the cathelicidins and defensins. Cathelicidins are constitutively expressed in the urinary tract and the expression of LL-37 (human cathelicidin) and cathelin-related antimicrobial peptide (CRAMP; murine homolog of LL-37) increases during UTI (Chromek et al., 2006). Cathelicidins are first produced by the uroepithelial cells, while later in infection infiltrating immune cells become the main producer of these antimicrobial peptides (Chromek et al., 2006). Cathelicidins are an important defense mechanism against UTI as CRAMP-deficient mice are more susceptible to UPEC infection (Chromek et al., 2006). Mice deficient in *defb1* (the murine homolog of human beta-defensin) display a higher incidence of spontaneous bacteriuria (Morrison et al., 2002), however, *defb1* deficiency did not impact UPEC clearance during UTI, suggesting that this antimicrobial peptide is dispensable during infection (Becknell et al., 2013). Another host strategy to fight UPEC colonization includes the expression of Tamm-Horsfall protein (THP), also known as uromodulin, in the urinary tract. THP binds to type 1 fimbriae and limits the interaction of bacteria with host receptors to prevent bacteria invasion (Zasloff, 2007). THP-deficient mice possess an impaired capacity to clear UPEC during UTI compared to WT mice. Additionally, it was observed that THP-deficient mice died during the course of the infection, underlining the importance of this defense molecule (Bates et al., 2004).

During bacterial colonization, umbrella cells undergo apoptosis, resulting in exfoliation of the bladder surface, which has been proposed to be a defense mechanism to eliminate infected

cells (Mulvey et al., 1998). Only 6 hours after infection, significant exfoliation leads to the loss of umbrella cells and exposure of underlying, less differentiated uroepithelial cells (Mulvey et al., 1998). It has also been reported that UPEC suppresses NF- κ B activity, which may enhance apoptosis of uroepithelial cells, as NF- κ B activation leads to anti-apoptotic effects (Klumpp et al., 2001). Additionally, a mechanism by which uroepithelial cells are able to expel UPEC was reported (Miao et al., 2015). The authors of this study observed that when UPEC invades uroepithelial cells, the bacteria are targeted by autophagy. However, they can neutralize lysosomal pH to prevent their own degradation. Lysosome neutralization is sensed by the cell and lysosome exocytosis is induced, which results in the expulsion of the intracellular bacteria (Miao et al., 2015).

Sensing of bacteria by uroepithelial and immune cells alerts the host to danger and triggers an immune response. During UTI, the pattern recognition receptors TLR4, TLR5, and TLR11 recognize the bacteria (Ragnarsdottir et al., 2008). TLR4 stimulation in wild type (WT) mice results in the activation of NF- κ B and subsequently, the expression of proinflammatory genes including IL-8 and IL-6 (Fischer et al., 2006). When TLR4 KO mice are infected with UPEC, they present higher bacterial burdens and bacterial clearance is impaired (Ashkar et al., 2008). Additionally, it was demonstrated, through the use of chimeric mice, that TLR4 expression is necessary in both stromal and hematopoietic cells to mount a sufficient inflammatory response and clear UPEC (Schilling et al., 2003). In a murine model of UTI, the absence of TLR5 resulted in decreased inflammation compared to WT mice early after UPEC infection and less efficient bacterial clearance (Andersen-Nissen et al., 2007). Finally, mice lacking TLR11 are also more susceptible to UPEC infection, however this TLR is not functional in humans due to the presence of an early stop codon (Zhang et al., 2004).

A strong innate immune response is triggered during UTI

During UTI, production of chemokines and cytokines is upregulated in the bladder mucosa in response to UPEC (Agace et al., 1993; Ingersoll et al., 2008; Samuelsson et al., 2004). UPEC infection induces a rapid infiltration of neutrophils that is mediated by IL-8 (Godaly et al., 2001; Godaly et al., 1997). The primary function of these cells is to phagocytize and kill invading pathogens during infection. CXCR1, the IL-8 receptor, is fundamental for the migration and activation of neutrophils (Freundt et al., 2000; Godaly et al., 2000; Hang et

al., 2000). During UTI, mice lacking this receptor display reduced neutrophil infiltration, higher titers of UPEC, and progression to bacteremia as well as renal scarring compared to WT mice (Freundt et al., 2000; Godaly et al., 2000; Hang et al., 2000). In addition to neutrophils, monocytes also infiltrate the bladder during UTI (Engel et al., 2008; Ingersoll et al., 2008). Monocytes are mononuclear phagocytes found in circulation that infiltrate inflamed tissues to differentiate to macrophages or dendritic cells (DCs). These cells can be divided into two subsets depending on their gene and protein expression patterns: classical (formerly known as inflammatory) and non-classical monocytes (Chow et al., 2011; Shi and Pamer, 2011; (Geissmann et al., 2003; Ingersoll et al., 2010). Classical monocytes express high levels of Ly6C and CCR2, are highly infiltrative, and have antimicrobial roles, while non-classical monocytes display low levels of Ly6C and CCR2 and are involved in tissue repair and patrolling (Auffray et al., 2007; Serbina et al., 2008; Shi and Pamer, 2011). We recently reported that when monocytes infiltrate the bladder during infection, the majority differentiate to macrophages while a small percentage of infiltrating monocytes differentiate to DCs (Mora-Bau et al., 2015). Depletion of neutrophils and classical monocytes together, significantly impairs bacteria clearance in the bladder and kidneys (Haraoka et al., 1999). Surprisingly, blocking neutrophil infiltration into the bladder, by neutralizing G-CSF, results in improved UPEC clearance (Ingersoll et al., 2008). These results may be due to an increase in macrophage activating cytokines, as G-CSF neutralization led to an increase of MCP-1, CCL-2, and IL-1 β in this study (Ingersoll et al., 2008). We recently observed that monocyte depletion in WT mice modestly improves bacterial clearance during primary UPEC infection, however we did not observe differences in bacterial clearance when infecting CCR2-deficient mice (which have greatly reduced numbers of circulating monocytes) compared to WT mice (Mora-Bau et al., 2015).

The role of other innate immune cells during UTI is not well described. However, a recent study demonstrated that innate immune cell crosstalk is necessary for a coordinated innate response, whereby resident macrophages attract monocytes from circulation, which differentiate to macrophages once in the bladder (Schiwon et al., 2014). These monocyte-derived macrophages produce TNF- α , which induces resident macrophages to produce CXCL2. CXCL2 induces MMP9 expression in neutrophils, in turn facilitating their transurothelial migration (Schiwon et al., 2014). This mechanism likely works in concert with cytokine and chemokine expression from the infected uroepithelium, which is known to mediate neutrophil recruitment and transurothelial migration (Godaly et al., 2000). Despite

their role in immune cell recruitment (Schiwon et al., 2014), we recently reported that macrophages are dispensable for UPEC clearance early in infection, as depletion of these cells did not affect bacterial burden in the bladder 24 hours post-infection (Mora-Bau et al., 2015). Additionally, a recent study proposed a role for bladder-resident mast cells, whereby these cells limit the generation of adaptive immunity during UTI by maintaining a state of immune privilege in the bladder through IL-10 secretion (Chan et al., 2013). This mechanism may not be universal, as IL-10 expression is variable in UTI. Some studies report IL-10 protein or mRNA expression (Duell et al., 2013; Duell et al., 2012) while others have failed to detect its presence during infection (Ingersoll et al., 2008; Mora-Bau et al., 2015). The reason for this discrepancy could be the use of different bacteria strains among the studies. In addition, we observed only a very small number of mast cells resident in the bladder (Mora-Bau et al., 2015). Finally, $\gamma\delta^+$ T cells may also play a role during UTI. These cells, which are resident in the bladder (Mora-Bau et al., 2015), are the main source of secreted IL-17A during UPEC infection. Mice lacking IL-17A showed deficient cytokine transcript upregulation and immune cell infiltration during UTI, resulting in an impaired clearance of UPEC (Sivick et al., 2010). Of note, the absence of IL-17A did not impact the protective immune response generated during UTI as IL-17A-deficient mice were as efficient as WT mice at clearing UPEC after a challenge infection (Sivick et al., 2010).

The adaptive immune response during UTI: the mystery starting to be solved

While the innate immune response during UTI has been extensively described, there is little information regarding the role of the adaptive immune response during this infection. Notably, the induction of a proper adaptive immune response is the basis for vaccine development and the more that is known about how these responses are generated, the better are the chances to generate efficient vaccines. Surprisingly, at the time we started this project, it was not entirely clear if an adaptive immune response was even generated during UTI. A significant part of our studies have concentrated on the demonstration that an intact adaptive immune system is necessary to respond against a challenge infection in a murine model of UTI (Mora-Bau et al., 2015). Many important questions still remain open in the field of adaptive immunity and UTI, such as: How this adaptive immune response is generated? Which cell subsets (*e.g.*, DCs subsets) participate in the generation of an adaptive immune

response against UPEC? What kind of effector cells are induced? And very importantly: can we manipulate and improve this response? Pointing out the paucity of information in the UTI field regarding the role of adaptive immunity in this infection, Box 1 exemplifies statements commonly found in UTI-related literature (see Box 1 below).

Box 1: What is the adaptive immune response during UTI? A common question in the field.

- “Existing data regarding the adaptive immune responses to UPEC are relatively **limited**”

Sivick and Mobley, 2010

- “A **more detail** knowledge of adaptive immune response to UPEC is a **prerequisite** for the development of next-generation candidate vaccines”

Hustand and Justice, 2010

- “In general, data regarding the adaptive immune response to UTI are **limited**” “It is **unclear** if the immune response is skewed toward a Th1-mediated or Th2-mediated response, and the role of Treg cells has not been elucidated”

Nielubowicz and Mobley, 2010

- “**Further work is needed** to understand the role of lymphoid cells in innate and adaptive immunity of the urinary bladder”

Hannan et al., 2012

- “**Many important questions** about the generation of these (adaptive) responses **remain unanswered**: How do antigen-presenting cells present pathogen antigens? Are UPEC-specific T cells activated? What kind of immunological memory is generated? Can memory T cells be generated to achieve sterilizing immunity?”

Ingersoll and Albert, 2013

To achieve an adaptive immune response against pathogens, different key players are required. Antigen presenting cells (APCs), characterized by the expression of MHC II molecules, are fundamental. These cells induce a humoral (antibody-mediated) and/or cellular immune (T cell-mediated) response, depending on the nature of the pathogen. APCs include macrophages, B cells, and DCs and are able to mediate immune responses by capturing, processing, and presenting antigens to effector cells, such as T cells. DCs are known as the orchestrators of the immune system, as they mediate communication between the innate and adaptive immune responses and are proposed to be the only cells able to prime naïve T cells (Buckwalter and Albert, 2009). They phagocytize pathogens and infected or transformed cells, sense the microenvironment, and migrate to the lymph node (LN) to deliver information to naïve lymphocytes. Depending on their origin, function, and distribution, DCs can be classified into different subsets (Kushwah and Hu, 2011). These DC subsets express different defining cell surface markers. In mice, LN-resident DC subsets can be distinguished by expression of CD4 and CD8 α molecules (Heath and Carbone, 2009; Turley et al., 2010). CD8 α ⁺ DCs have a greater capacity for cross-presenting antigens to CD8⁺ T cells, while CD8 α ⁻ DCs (including CD4⁺CD8 α ⁻ and CD4⁻CD8 α ⁻ DCs) are thought to be more efficient at presenting antigens to CD4⁺ T cells (Dudziak et al., 2007). In the periphery, at least two populations of DCs can be identified based on their expression of CD11b and CD103 molecules (Moore and Anderson, 2013). CD103⁺ DCs are considered to be the equivalent to the CD8 α ⁺ DCs present in LNs, as they share several characteristics (Bedoui et al., 2009; Dudziak et al., 2007). CD8 α ⁺ DCs and CD103⁺ DCs are both highly efficient in cross-presenting antigens and therefore inducing a CD8⁺ T cells response (Bedoui et al., 2009; Dudziak et al., 2007). Additionally, CD8 α ⁺ DCs and CD103⁺ DCs rely on the same transcription factors for their development - basic leucine zipper transcription factor ATF-like 3 (BATF3), inhibitor of DNA protein 2 (ID2) and interferon-responsive factor 8 (IRF8) (Edelson et al., 2010; Jackson et al., 2011). Batf3 is fundamental for the development of CD8 α ⁺ DCs and CD103⁺ DCs, as mice lacking this protein also lack both of these DC subsets and have an impaired CD8⁺ T cell response (Hildner et al., 2008). Early in 1980, MHC II⁺ cells were described to exist in naïve bladders of human, rat, guinea-pig and mouse (Gardiner et al., 1986; Hart and Fabre, 1981a; Hjelm et al., 1982), however due to the limited number of cell markers used in these studies, it is challenging to conclude if these cells are DCs or also include macrophages. Importantly, we detected DCs in naïve bladders during the course of our studies, characterized by the expression of CD45, MHC II, CD11c, CD11b and CD103

(Miller et al., 2012; Mora-Bau et al., 2015). We described that, as well as in other mucosa organs (Neyt and Lambrecht, 2013; Rescigno, 2010), the bladder contains the CD11b⁺ and CD103⁺ DC subsets (Mora-Bau et al., 2015). It was reported that DCs are dispensable to clear UPEC early in infection (Engel et al., 2006); however, we found that DCs are required to induce an adaptive immune response during UTI as mice only partially depleted of DCs exhibit compromised clearance during challenge infection (Mora-Bau et al., 2015).

In contrast to DCs, macrophages are not thought to play a significant role in priming naïve T cells and inducing an adaptive immune response (Buckwalter and Albert, 2009; Hashimoto et al., 2011). However, it is possible to find literature reporting that macrophages can prime CD4⁺ and CD8⁺ T cells, *in vitro* and *in vivo* (Asano et al., 2011; Bernhard et al., 2015; Pozzi et al., 2005). Macrophages can, however, modulate the induction of adaptive immunity in other ways, such as by cytokine production (Schreiber et al., 2013; Silva, 2010). For example, during *Citrobacter rodentium* infection, macrophages in the gut secrete significant amounts of IL-12, which biases the generation of an adaptive immunity toward a Th1 response (Schreiber et al., 2013). Macrophages can also modulate adaptive immunity through antigen sequestration (Jakubzick et al., 2006; Kradin et al., 1999; MacLean et al., 1996). It has been shown that after injecting mice intratracheally with heat-killed *Listeria*, macrophages take up a majority of the antigen, limiting acquisition by DCs and that an adaptive immune response is achieved only after macrophage depletion (MacLean et al., 1996). Moreover, we demonstrated that macrophages negatively impact the generation of an adaptive immune response during UTI likely by limiting DC access to UPEC (Mora-Bau et al., 2015).

B and T cells are the effector cells of the adaptive immune system; they specifically recognize pathogens and infected cells through antigen-specific receptors and confer protection against future infections. After antigen encounter, B cells differentiate to memory B cells or plasma cells (Figure 7). Memory B cells are long lasting and during secondary infection, they are quickly reactivated and differentiate to plasma cells. Newly generated plasma cells aid in the recovery during primary infection through antigen-specific immunoglobulin secretion (Nothelfer et al., 2015). Antibodies mediate the clearance of extracellular pathogens by activating the complement cascade, by direct neutralization, or by interacting with other immune cells (binding to Fc receptors). The induction of antigen-specific antibodies as a way to protect individuals from reinfection is the hallmark of efficient vaccination. There are different classes of antibodies including IgG, IgM, IgA, IgD, and IgE; antibody class is

defined by the heavy chain constant region of the immunoglobulin and determines the effector function of the antibody. IgA is the most abundant antibody in mucosal secretions, providing first line immune protection at mucosal surfaces (Macpherson et al., 2008). Secretory IgA has been observed in the bladder during UTI (Shi and Pamer, 2011; Svanborg-Eden and Svennerholm, 1978). There is evidence that a humoral response arises during UTI as UPEC-specific antibodies have been detected during infection in humans, non-human primates, and mice, and can inhibit UPEC binding to uroepithelial cells *in vitro* (Hopkins et al., 1987; Svanborg-Eden and Svennerholm, 1978; Thumbikat et al., 2006). Additionally, as discussed above, different vaccination strategies have been tested to treat UTI and some of them induce a humoral response in mice, non-human primates, and humans (Asadi Karam et al., 2013; Czerwionka-Szaflarska and Pawlowska, 1996; Langermann et al., 2000; Langermann et al., 1997).

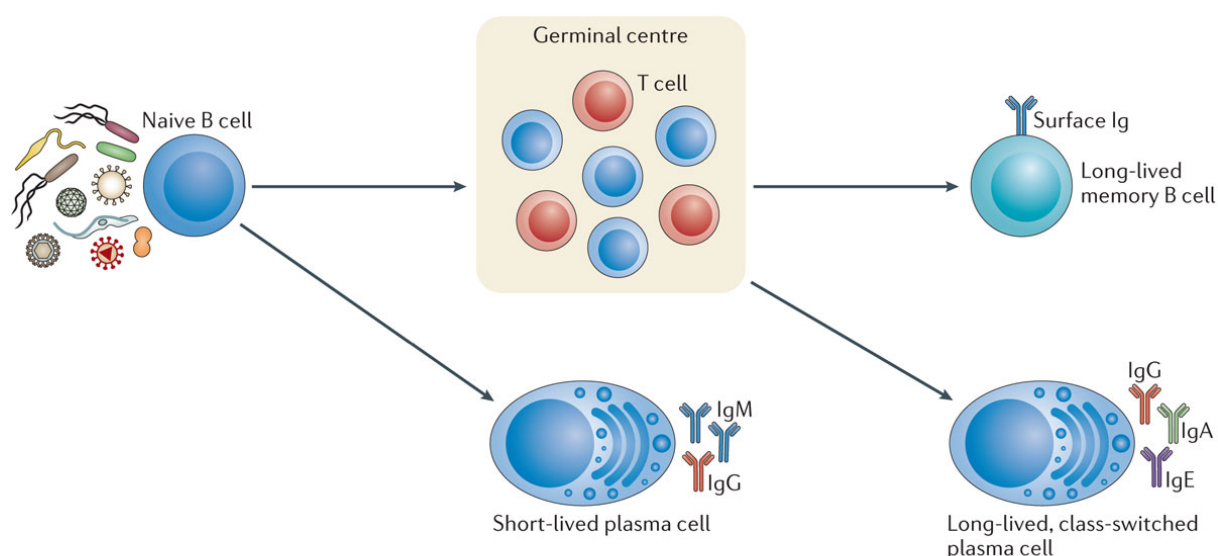


Figure 7: B cell immune response. In response to activation signals, naive mature B cells proliferate and differentiate into effector cells. B cell activation results from the integration of several infection-related signals, including binding of specific antigens to the B cell receptor (BCR) and pattern recognition receptor (PRR) ligands. In an early polyclonal response, short-lived plasma cells that secrete polyreactive antibodies can be generated. Sustained B cell activation leads to further differentiation and selection in organized lymphoid structures, called germinal centers (GCs). The activation of nuclear factor- κ B (NF- κ B) and upregulation of activation-induced cytidine deaminase (AID) induce affinity maturation of antibodies through somatic hypermutation and class-switch recombination of the antibody heavy chain. This ultimately results in the differentiation of specific, long-lived plasma cells and memory B cells, which confer protective immunity. Ig: immunoglobulin. Figure and legend modified from Nothelfer et al., 2015.

T cells can be generally divided into T “helper” and “cytotoxic” cells to identify CD4⁺ and CD8⁺ T cells, respectively. The main function of T helper (Th) cells is to support the immune response by secretion of cytokines and chemokines to activate neighboring cells and/or to recruit more immune cells to the site of inflammation/infection. On the other hand, the main function of cytotoxic T cells is to directly kill infected cells, although they are also capable of producing a diverse range of cytokines. There is, however, evidence that CD4⁺ T cells can also be cytotoxic and eliminate tumor cells and virally infected cells (Fang et al., 2012; Quezada et al., 2010). Additionally, CD8⁺ T cells can act as “helper” instead of cytotoxic by secreting cytokines and collaborating with CD4⁺ T cells in asthma and autoimmune encephalomyelitis (Huber et al., 2013; Huber and Lohoff, 2015; Viseruna et al., 2013).

Upon activation by DCs, CD4⁺ T cells differentiate into Th cell subsets with distinct effector functions and cytokine profiles. The different subsets of Th cells include Th1, Th2, and Th17 T cells, among others. Th1 cells are characterized by the production of IFN- γ and mediate cellular immunity, while Th2 cells produce IL-5, IL-4, and IL-13 and mediate humoral immunity. Th17 produce IL-17, IL-21, and IL-22. Th17 T cells can be pro-inflammatory and play a role in host defense against infection (Korn et al., 2009; Peck and Mellins, 2010). Additionally, Th17 can also promote autoimmune diseases (Koenders et al., 2005; Langrish et al., 2005). Naïve T cells develop into one Th cell subset or another depending on the signals they receive from DCs during the T cell priming phase in the LNs. Generally speaking, DCs producing IL-12 induce naïve T cells to acquire a Th1 phenotype, whereas DCs producing IL-4 drive T cell differentiation towards a Th2 phenotype. The Th17 subset is driven by the cytokines IL-6, IL-23, TGF- β , IL-1, and IL-21 (Korn et al., 2009).

There is little evidence that a cellular response develops during UTI and this area remains poorly described. Thumbikat et al. used UPEC expressing ovalbumin (OVA) to infect mice and evaluate the adaptive immune response generated during infection. The authors observed T cell infiltration into the bladder during the course of the infection, as well as T cell activation and cytokine secretion during *ex vivo* OVA stimulation (Thumbikat et al., 2006). Additionally, OVA-specific antibodies were detected in the serum of infected animals. Finally, the authors also demonstrated that transfer of serum or T cells from infected animals limited infection in naïve mice (Thumbikat et al., 2006). Additionally, T cell depletion during UTI impairs UPEC clearance during a challenge infection (Mora-Bau et al., 2015). Interestingly, we observed that bacterial clearance is equivalent between WT and Batf3

deficient mice during UPEC challenge, suggesting that although T cells are needed for a response against UPEC, CD8⁺ T cells, specifically, are dispensable during UTI (Mora-Bau, unpublished). However, there is no evidence regarding which T cell subsets are generated during UTI and further studies are needed to advance our knowledge regarding the cellular response during UTI.

The high rates of recurrence in UTI patients suggest a defect exists in the generation of an adaptive immune response during this infection. In the context of UTI, it was recently suggested that mast cell-derived IL-10 impedes the generation of a cellular and humoral-mediated immune response against UPEC (Chan et al., 2013). We might hypothesize that the bladder represents an immunoprivileged site, however there is evidence showing that it is possible to induce an efficient adaptive immune response from the bladder mucosa (Biot et al., 2012 ; Ratliff, 1992; Ratliff et al., 1993). *Bacillus Calmette-Guerin* (BCG) (an attenuated vaccine strain of *M. bovis* generated to prevent tuberculosis) intravesical instillation has been used for almost 40 years to treat non-muscle invasive bladder cancer (NMIBC) with high rates of success (Herr and Morales, 2008; Redelman-Sidi et al., 2014). BCG instillation into the bladder, induces the generation of BCG-specific CD8⁺ T cells, which have been shown to be critical for antitumor immunity (Biot et al., 2012; Ratliff et al., 1993). Additionally, it has been demonstrated that the depletion of either CD4⁺ or CD8⁺ T cells abolishes BCG-mediated antitumor activity (Ratliff et al., 1993). Moreover, it is noteworthy to consider that despite high rates of infection, around 50% of patients experiencing one UTI will not experience recurrence, suggesting that they are able to develop an efficient adaptive immune response.

Aims of our study

We are interested in how an adaptive immune responses are induced during UTI. To develop this project, we first schematized a model that, in a general and simplified way, includes the steps necessary to generate an adaptive immune response during UPEC infection (Figure 8). During UTI, UPEC (or another uropathogen) invades the bladder mucosa and induces an inflammatory response characterized by cytokine secretion and innate immune cell infiltration, as described above. Early during infection, UPEC is captured by different immune cells (including APCs) in the bladder, which is a fundamental step for the initiation

of adaptive immunity (antigen uptake, Figure 8). To induce an adaptive immune response against UPEC, DCs should acquire bacteria, migrate to LNs (APC migration, Figure 8), and present bacterial antigens to naïve T and B cells (effector response induction, Figure 8). Then, effector cells should migrate to the bladder and clear the infection (effector cell migration, Figure 8). Surprisingly, at the beginning of our study, none of the proposed steps to induce adaptive immunity during UTI were described, and we first focused our attention on the early events occurring during infection - namely, UPEC acquisition in the bladder. We were interested in defining whether an adaptive immune response existed during UTI as well as the key players to initiate this response. Additionally, as the rates of UTI recurrence are high, we also aimed to decipher if a defect in the induction of adaptive immunity could be revealed.

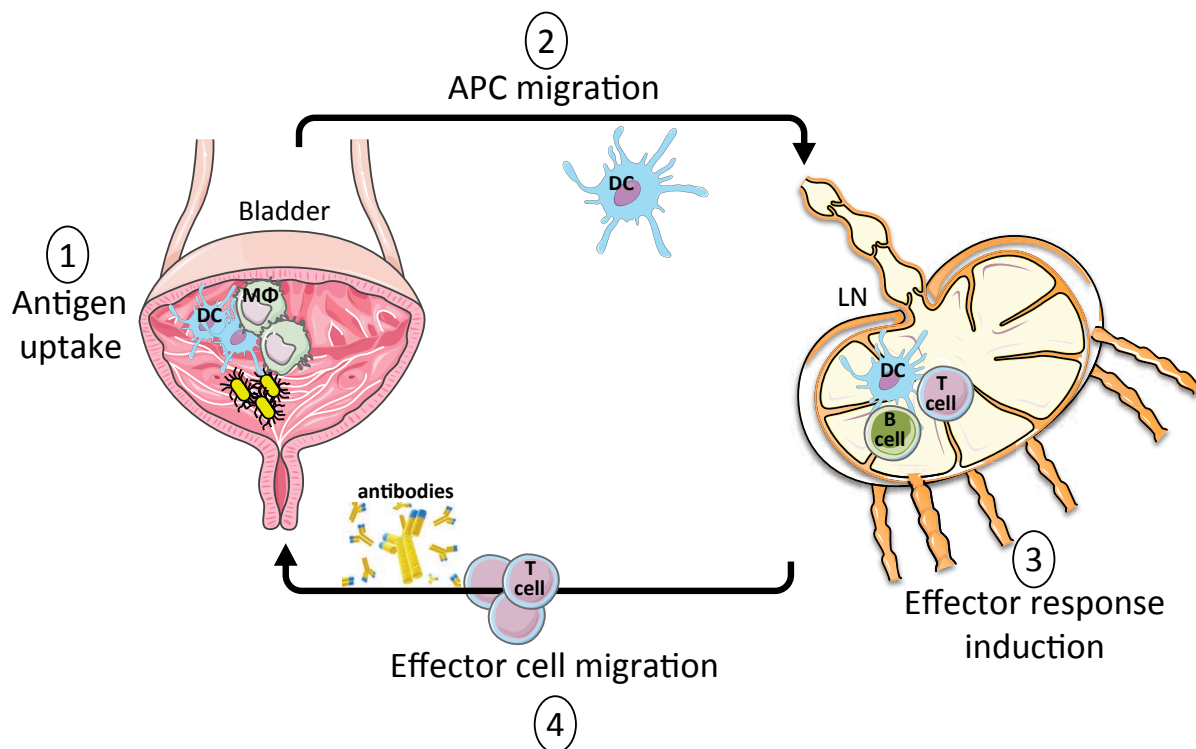


Figure 8: Proposed model for the generation of an adaptive immune response during UTI. In this scheme, we illustrate the different required steps to induce adaptive immunity during UTI including: (1) antigen acquisition in the bladder, (2) DC migration to draining LNs, (3) priming of naïve lymphocytes, and (4) migration of effector cells to the bladder.

Chapter 2: Deciphering the early events leading to an adaptive immune response in UTI

Introduction

The results presented in this chapter are part of the manuscript entitled “Macrophages Subvert Adaptive Immunity to Urinary Tract Infection” published in Plos Pathogens (Mora-Bau et al., 2015). Portions of the manuscript have been reproduced here, including the introduction.

It is estimated that one out of two women will experience UTI during their life time and nearly half of all women infected will suffer recurrence (Foxman, 2010). Currently, there is little consensus in the field regarding the underlying causes of the high rate of recurrence. Mechanisms previously proposed to explain this phenomenon include that UPEC forms protected reservoirs in the bladder, reemerging at later time points after initial infection (Anderson et al., 2004a; Mysorekar and Hultgren, 2006); that UPEC strains colonize the gut and periodically migrate to the urinary tract (Chen et al., 2013a); or that the immune response to infection is suppressed by mast cell-derived IL-10 in the bladder (Chan et al., 2013).

Events occurring early in the bladder during infection are key for the development of an adaptive immune response during UTI. The innate immune response to UPEC infection is characterized by robust cytokine and chemokine expression, leading to rapid neutrophil and monocyte infiltration and subsequent bacterial clearance (Engel et al., 2008; Godaly et al., 2000; Haraoka et al., 1999; Shahin et al., 1987). Depletion of both neutrophils and classical monocytes, by Gr1 antibody treatment, leads to increased bacterial burden (Daley et al., 2008), whereas a reduction in circulating neutrophils alone decreases bacterial burden, suggesting that monocytes help eliminate bacteria in the bladder (Haraoka et al., 1999).

The mechanisms involved in the initiation of adaptive immunity, and indeed the full nature of the response generated from the bladder during UTI, remain unclear. The majority of studies have focused on innate immunity to UTI, such as neutrophil or monocyte infiltration, while only a limited number of studies have focused on adaptive immune mechanisms. For example, UPEC-specific antibodies arise during UTI in mice, non-human primates, and human patients, and can inhibit UPEC binding to uroepithelial cells *in vitro* (Hopkins et al., 1987; Svanborg-Eden and Svennerholm, 1978; Thumbikat et al., 2006). With respect to the role of effector cells, only one study has examined the induction of antigen-specific antibody and T cell responses after UPEC infection, demonstrating that transfer of serum or T cells from infected animals limits infection in naïve mice (Thumbikat et al., 2006).

Our work has focused on the initiation of adaptive immunity to UPEC to determine what events or mechanisms exist that prevent the induction of sterilizing immunity. Here, we have demonstrated the necessity of the adaptive immune system to respond to UPEC challenge. Additionally, we also have shown that macrophages are the main cell type among the bladder APCs to capture UPEC early in infection. Strikingly, macrophage depletion, prior to primary infection, improves adaptive immune responses to challenge infection in a macrophage-replete environment. These data support a model in which bladder-resident macrophages sequester bacteria, consequently limiting adaptive immune responses, and provide an explanation for the failure of the immune system to respond effectively to UPEC infection.

Generation of fluorescent UPEC strains

To study the immune response generated during UTI, we developed bacteria expressing antibiotic cassettes and the fluorescent proteins RFP (UTI89-RFP, kan^R) or GFP (UTI89-GFP, amp^R) in collaboration with Dr. Ghigo's laboratory. These strains were engineered using lambda red recombination (Chaverocche et al., 2000) to introduce an *aphA-marsRFP* or *bla-GFP* cassette in the UTI89 chromosome at the *attB* lambda phage integration site. The parental strain UTI89 is sensitive to antibiotics, UTI89-RFP is resistant to kanamycin, and UTI89-GFP is resistant to ampicillin.

Our goal in producing these strains was to follow the fate of the bacteria by flow cytometry. Prior to their creation, we had attempted to perform this experiment with an existing UTI89-GFP strain, generated in the laboratory of Scott Hultgren (Wright et al., 2007). Mice were infected and, at different time points post-infection (P.I.), bladders were obtained and analyzed by flow cytometry. However, it was not possible to reliably identify GFP⁺ cells in the bladder (UPEC-containing cells) due to the autofluorescence of bladder macrophages and uroepithelial cells (Figure 9A). To overcome this challenge, we generated new strains and were able to successfully follow UTI89-RFP to evaluate UPEC acquisition in the bladder. Importantly, these are isogenic strains, meaning they are identical in every respect except for their insertion.

UTI89-RFP and UTI89-GFP fluorescence was evaluated by microscopy (Figure 9B). To evaluate the infectivity of the new fluorescent strains, we performed *in vitro* invasion assays with the mouse uroepithelial cell line NUC-1 (De Boer et al., 1993). We observed that UTI89-

GFP and UTI89-RFP were able to invade uroepithelial cells with the same efficiency as the parental UTI89 strain (Figure 9C). Additionally, to evaluate *in vivo* infectivity, mice were infected with 10^7 colony-forming units (CFU) of UTI89, UTI89-GFP, or UTI89-RFP and sacrificed 24 hours later to evaluate bacterial load. We observed that UTI89-GFP and UTI89-RFP had similar infectivity *in vivo* compared to the parental UTI89 strain (Figure 9D).

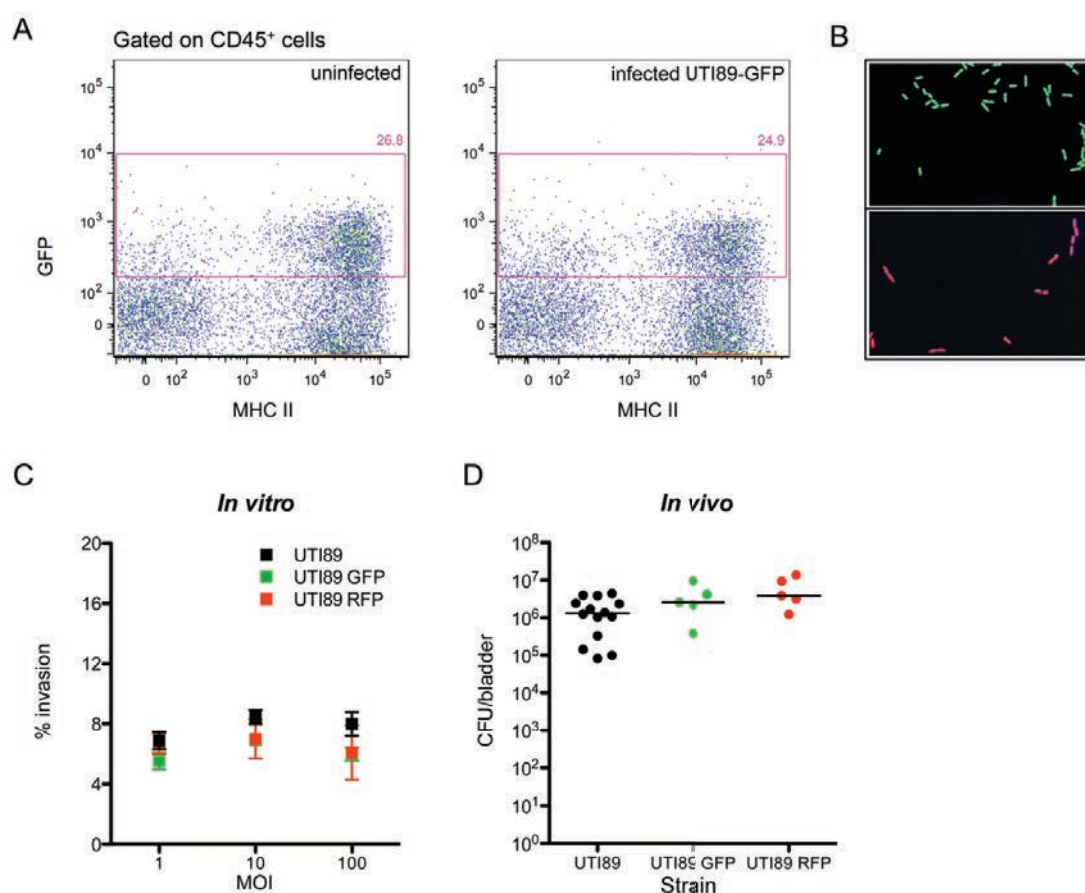


Figure 9: Fluorescent UPEC strains. (A) Cytometry plots, gated on all CD45⁺ cells, depict GFP fluorescence (gated in pink with percentages) in mice either uninfected or infected with UTI89-GFP at 4 hours post-infection. (B) Fluorescence of UTI89-GFP and UTI89-marsRFP was confirmed by microscopy. (C) The mouse uroepithelial cell line, NUC-1, was infected with the parental UTI89, UTI89-GFP, or UTI89-RFP at an MOI of 1,10, or 100. Cells were lysed and bacterial titers determined by serial dilution 30 minutes P.I. The percentage of invasion refers to the number of bacteria obtained after infection x 100/number of bacteria in the inoculum. (D) Mice were instilled with 1×10^7 CFU of UTI89, UTI89-GFP, or UTI89-RFP. CFU per bladder were determined by serial dilution at 24 h P.I. Each dot represents one mouse. Experiments were repeated 2 times.

UTI challenge infection model

To evaluate the role of adaptive immune components, such as B, T, and dendritic cells (DCs), during UTI, we amended an existing murine model of UPEC-induced cystitis to include challenge infection. Specifically, female mice were intravesically instilled with 10^7 CFU of UPEC isolate UTI89 (Hung et al., 2009), resistant to either ampicillin or kanamycin. The urine was monitored every 3-5 days to evaluate the resolution of acute infection. Three to four weeks later, when mice had resolved the primary infection (defined by sterile urine), animals were challenged with 10^7 CFU of an isogenic UPEC strain, resistant to a different antibiotic compared to the strain used for the primary infection. Twenty-four hours post-challenge, mice were sacrificed and bacterial burden was evaluated (Figure 10). Notably, infecting the mice with isogenic UPEC strains, differing only by their antibiotic resistance, allows the differentiation between the quiescent bacterial reservoir established during the primary infection (Mysorekar and Hultgren, 2006) and the challenge strain. Importantly, this distinction has not been made in previous studies, therefore it was not possible to conclude if bacteria measured after challenge infection arise from the primary or the challenge infection or a mixture of both infections (Thumbikat et al., 2006).

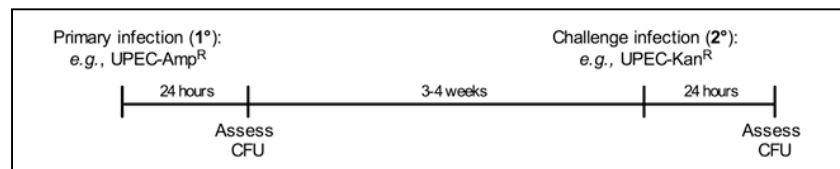


Figure 10: Challenge infection model. Experimental scheme used in the study. Mice were infected with 10^7 CFU of UPEC, resistant to ampicillin. A group of mice were sacrificed 24 hours later to evaluate CFU per bladder and another group was monitored every 5-7 days to determine the presence of bacteria in their urine. Once the infection resolved (defined by sterile urine), around 3-4 weeks P.I., mice were challenged with an isogenic strain of UPEC resistant to kanamycin. Animals were sacrificed 24 hours post-challenge to assess CFU per bladder. In the course of the study, we varied which bacterial strain was used for the primary or challenge infection, ruling out that the order of infection impacted our results.

UPEC infection induces an adaptive immune response mediated by DCs

Although surprising, the necessity of the adaptive immune system to respond to UPEC infection has never been evaluated. Female wildtype (WT) or RAG2^{-/-} mice were instilled

with 10^7 CFU of UTI89, resistant to either ampicillin or kanamycin. Twenty-four hours P.I. mice were sacrificed to evaluate bacterial titers in the bladder. Additionally, another group of mice remained alive and their urine was monitored to evaluate the resolution of acute infection. Once mice cleared the primary infection, animals were challenged with 10^7 CFU of an isogenic UPEC strain. Twenty-four hours post-challenge, mice were sacrificed and bacterial load was evaluated (Figure 11A). When comparing bacterial titers in WT mice sacrificed 24 hours post-challenge and post-primary infection, we observed a >2 log reduction in CFU after challenge infection (Figure 11A). By contrast, the bacterial burden after challenge infection in RAG2^{-/-} mice was comparable to that after primary infection (Figure 11A).

Next, we evaluated the role of DCs during UTI, as these cells prime naïve lymphocytes and initiate adaptive immune responses. To this end, we utilized CD11c-DTR chimeric mice. Chimeric mice were generated by irradiating WT mice with a single dose of 5-6 gray in an x-ray irradiator. These mice were reconstituted with total bone marrow cells from CD11c-DTR mice. Twelve weeks post-reconstitution, we depleted CD11c-expressing cells prior to primary infection, by administration of two doses of diphtheria toxin (DT) (Figure 11B). As has been previously reported (Probst et al., 2005), DT treatment impacted bladder resident macrophages as well (Figure 12), however this reduction was not significant as the number of macrophages present in DT-treated animals was within the range observed in naïve untreated animals (Table 2). Twenty-four hours after depletion, mice were infected as described in Figure 10 and urine was evaluated every 3-5 days to determine resolution of the infection. Once the primary infection was cleared, animals were challenged with an isogenic UTI89 strain, as described in Figure 10. Importantly, to determine bacterial burden after a primary infection specifically in the chimeric mice, an additional group of naïve and untreated chimeric animals received a primary infection at the same time as the infected mice received the challenge infection (Figure 11B, 1° group). Mice were sacrificed 24 hours post-primary or challenge infection and we observed that animals treated with PBS were better able to clear UPEC after challenge than DC-depleted mice (Figure 11B).

These results suggest that the improvement in bacterial clearance observed after a challenge infection is mediated by an adaptive immune response, dependent on DCs and lymphocytes. Interestingly, bacteria titers derived from the primary infections, representing the UPEC reservoirs, were comparable between WT and RAG2^{-/-} mice, as well as between PBS and DT-

treated animals, suggesting that reservoirs remain untouched during an adaptive immune response.

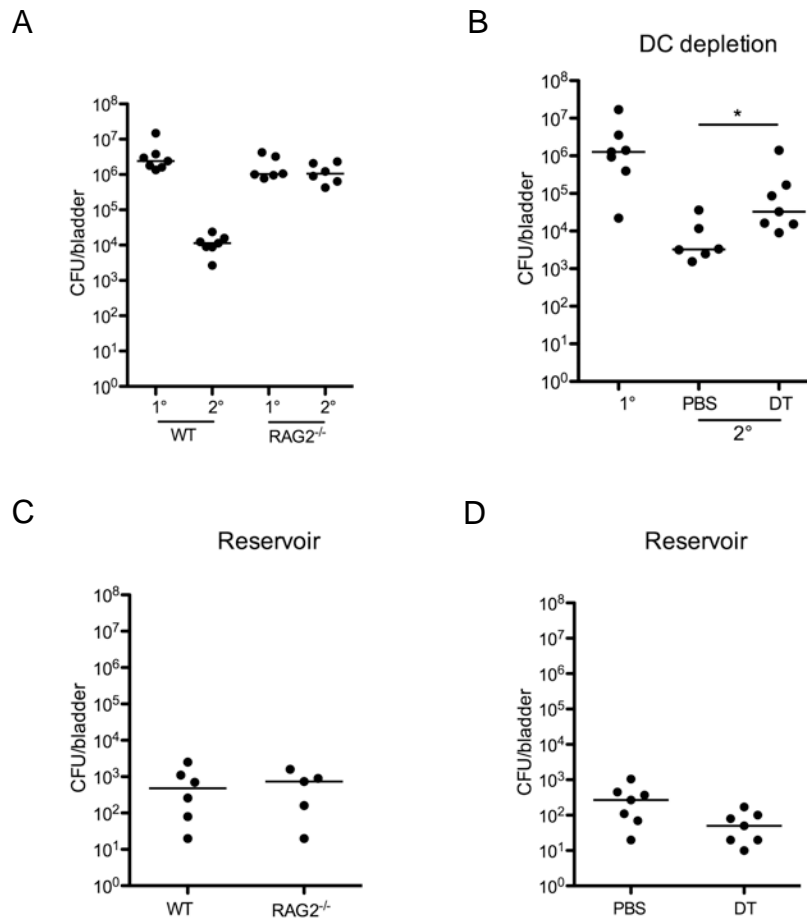


Figure 11: An adaptive immune response is necessary for bacterial clearance during UPEC challenge infection. (A) Female C57Bl/6 (WT) or RAG2^{-/-} mice were instilled with UTI89 and sacrificed 24 hours P.I. (1°) or challenged with an isogenic UPEC strain carrying a different antibiotic marker and sacrificed 24 hours P.I. (2°) to evaluate bacterial burden. (B) 12 weeks post-reconstitution, chimeric CD11c-DTR mice were treated with PBS or diphtheria toxin (DT) to eliminate DCs and infected with UTI89 24 hours post treatment. Mice were challenged with the isogenic UPEC strain and sacrificed 24 hours P.I. to measure CFU/bladder (2°). At the time of the challenge infection in (B), an additional group of naïve CD11c-DTR chimeric mice was infected with UTI89 to evaluate CFU after a primary UPEC infection (1°). (C-D) Graphs depict CFU/bladder of the primary strain from the infections in (A) and (B), respectively. Each dot represents one mouse, lines are medians. Experiments were performed 2 times with 5-7 mice per group in each experiment. *p=0.0221, Mann-Whitney.

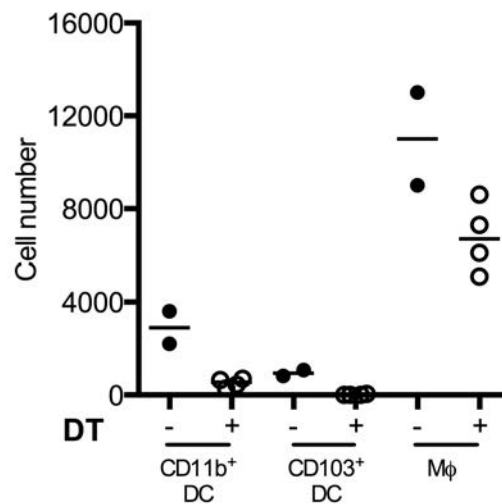


Figure 12: DT-mediated DC ablation. Irradiated C57Bl/6 mice were reconstituted with bone marrow from CD11c-DTR animals and allowed to rest for 12 weeks. Prior to infection, mice were treated two times with PBS (-) or 4 ng/g diphtheria toxin (+). 24 hours post-treatment, a cohort of animals were analyzed by flow cytometry to assess the extent of depletion in the bladder. Graph depicts the number of DCs in the bladder in PBS or DT treated mice. Each dot is one mouse, lines are medians. Depletion efficiency was tested in each batch of chimeric mice prior to experimentation, n=2-4 mice per group.

The steady state bladder contains a heterogeneous immune cell population

After demonstrating the necessity of the adaptive immune system to respond against a secondary UPEC infection, we were interested in how adaptive immune responses are initiated from the bladder mucosa. To accomplish this, we first defined the resident immune cell compartment of the bladder. While some immune cell populations have been described in naïve bladder, such as DCs and $\gamma\delta^+$ T cells (Christmas, 1994; Gardiner et al., 1986; Hart and Fabre, 1981a, b; Hjelm et al., 1982), a comprehensive analysis of all immune cell populations has not been performed. To define the immune cell populations residing in steady state bladder tissue, we evaluated the CD45⁺ cell compartment from naïve female mice. Bladders were enzymatically digested and the expression of different proteins (Table 3), commonly used to define immune cell populations, was evaluated by flow cytometry. APCs, defined as MHC II⁺, represented the majority of CD45⁺ cells (69% \pm 7.5 of CD45⁺ cells, Figure 13A, E). Among the APCs, macrophages, defined by CD64 and F4/80 co-expression (Gautier et al., 2012; Gordon et al., 2011; Tamoutounour et al., 2012) comprised the largest population

(~40% of CD45⁺ cells) (Figure 13B, E). The DC compartment contained CD11b⁺ and CD103⁺ DCs, corresponding to the 15% and 5% of CD45⁺ cells, respectively (Figure 13B, E). Within the MHC II⁺ CD11b⁺ gate, we identified CD11b^{low-int} cKit⁺ IgE⁺ mast cells (Jonsson and Daeron, 2012), NK1.1⁺ NK cells, CD3⁺ CD4⁺ and CD3⁺ $\gamma\delta$ ⁺ T cells (Figure 13C, E). Of note, we never observed CD8⁺ T cells in naïve bladders. It was recently shown that classical Ly6C⁺ monocytes are found in steady state within naïve nonlymphoid tissues, such as skin (Jakubzick et al., 2013; Tamoutounour et al., 2013). In line with these observations, we identified resident Ly6C⁺ monocytes as well as SiglecF⁺ eosinophils (Dyer et al., 2011) in the MHC II⁺ CD11b⁺ gate (Figure 13D, E). Notably, we did not observed neutrophils in naïve bladders.

Table 2: Immune cell populations in naïve bladders.

Naïve bladder	
Cell subset	Cell number ^a
CD45 ⁺	35714 ± 11161
Macrophages	9035 ± 3480
CD11b ⁺ DCs	4046 ± 1785
CD103 ⁺ DCs	1011 ± 443
Monocytes	720 ± 467

^a Cell numbers are displayed as the mean ± the standard deviation. Values are derived from at least 8 mice, analyzed in separate experiments.

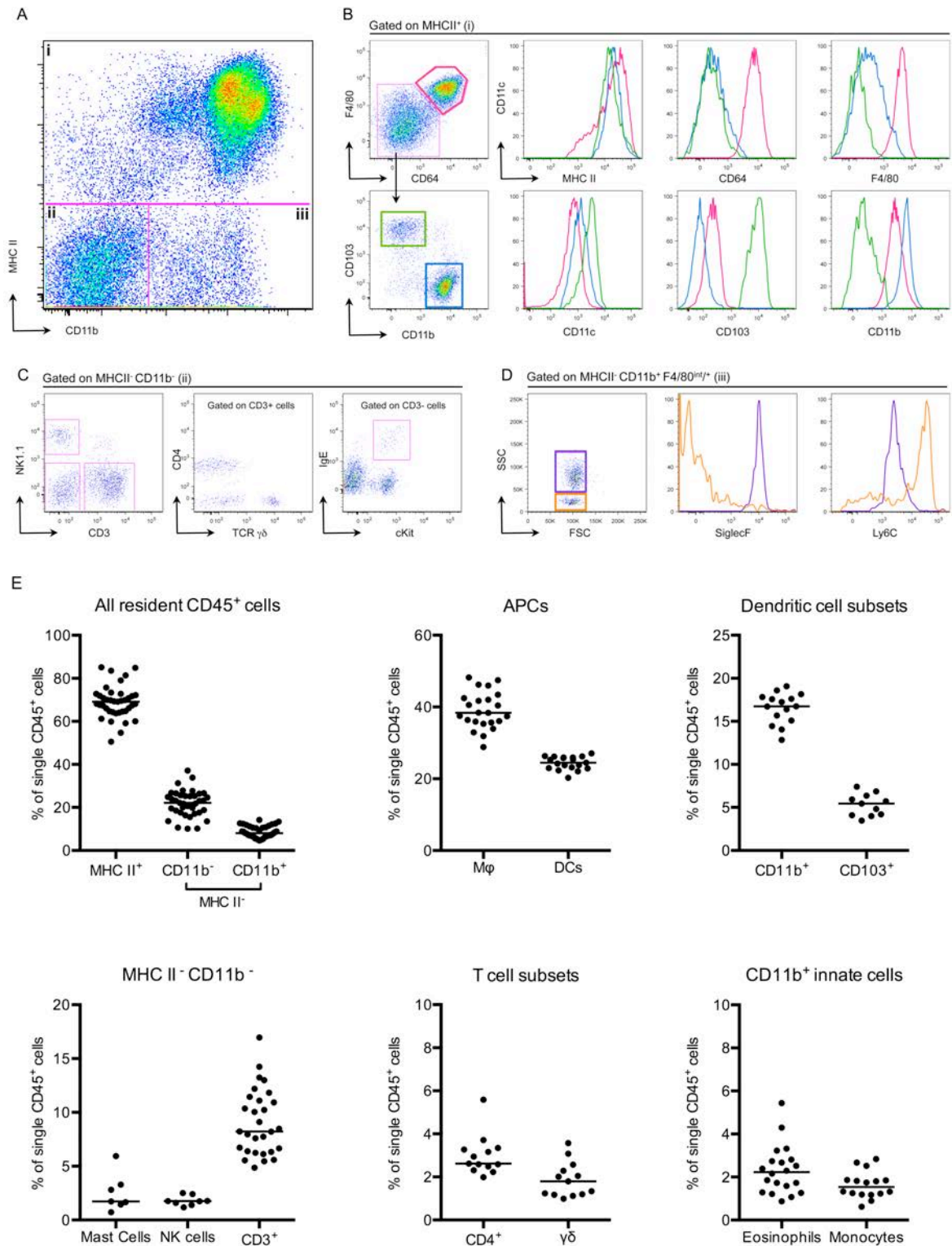


Figure 13: The bladder contains a diverse immune cell repertoire. Naïve bladders from female C57Bl/6 mice were digested for flow cytometry. Single cell preparations were stained with antibodies indicated in Table 3. (A) Single, CD45⁺ cells were gated into 3 groups (i-iii) according to their CD11b and MHC II expression levels. (B) MHC II⁺ cells from gate (i) were divided into 3 populations, F480⁺ CD64⁺ macrophages (pink gate), CD11b⁺ DCs (blue gate)

and CD103⁺ DCs (green gate). The expression levels of MHC II, CD64, F4/80, CD11c, CD103, and CD11b are depicted in the histograms (macrophages - pink lines, CD11b⁺ DCs - blue lines, CD103⁺ DCs - green lines). (C) MHC II⁺ CD11b⁺ cells from gate (ii) were subdivided by their expression of CD3 and NK1.1. CD3⁺ cells were divided into CD4⁺ and TCR α ⁺ and the CD3⁺ gate shows cKit⁺ IgE⁺ mast cells. (D) The dot plot, from gate (iii) depicts MHC II⁺ CD11b⁺ F480^{int/+} eosinophils (purple gate) and monocytes (orange gate). Histograms depict expression levels of SiglecF and Ly6C from gate (iii) (eosinophils - purple lines, monocytes - orange lines). (E) Graphs depict immune cell populations in the naïve bladder as percentage of all CD45⁺ cells. Representative cytometry dot plots and histograms from different individual bladders are depicted, with the exception of the mast cell plot in (C), which depicts three pooled bladders due to the low number of mast cells. In (E), each dot represents one bladder, lines are medians, plots are pooled from three or more experiments.

UPEC is acquired by macrophages early after infection

Having defined the composition of the steady state immune cell compartment of the bladder, we sought to determine which of these cell populations acquire UPEC early after infection. Antigen acquisition is a fundamental step during infection and development of an adaptive immune response. Mice were instilled with 10⁷ CFU of UTI89-RFP or UTI89 and sacrificed at 4, 24, and 48 hours P.I. to analyze their bladders by flow cytometry. UTI89-RFP-containing cells were identified by gating CD45⁺ cells with RFP fluorescence levels greater than those in bladders infected with the non-fluorescent UTI89 strain (Figure 14A) and their phenotypes were defined according to the expression of cell-specific markers as in Figure 13. At 4 hours P.I., the majority of UTI89-RFP⁺ cells localized to the MHC II⁺ cell compartment. At 24 and 48 hours P.I., UTI89-RFP⁺ cells were evenly distributed between the MHC II⁺ and II⁻ gates (Figure 14B). As our objective was to investigate initiation of adaptive immune responses, we focused our attention on APCs acquiring UPEC in the bladder (UPEC⁺ MHC II⁺ cells). Among this compartment, we observed that the majority displayed a macrophage phenotype (MHC II⁺ CD64⁺ F4/80⁺) (Figure 14C). Notably, two subpopulations were distinguishable in the macrophage gate at 24 and 48 hours P.I., representing resident (CD64^{hi} F4/80^{hi} Ly6C⁻) and monocyte-derived macrophages (CD64^{int} F4/80^{int} Ly6C⁺) (Figure 14C). Importantly, while the number of UPEC-containing DCs changed very little during the course of infection, the number of macrophages harboring UTI89-RFP increased more than 7-fold at 24 hours and remained elevated at 48 hours (Figure 14D). At all the evaluated timepoints,

macrophages harbored approximately 60-80% of the bacteria found within the APC compartment, demonstrating that they were the main cell type within this compartment to phagocytose UPEC at early timepoints post-infection (Figure 14E).

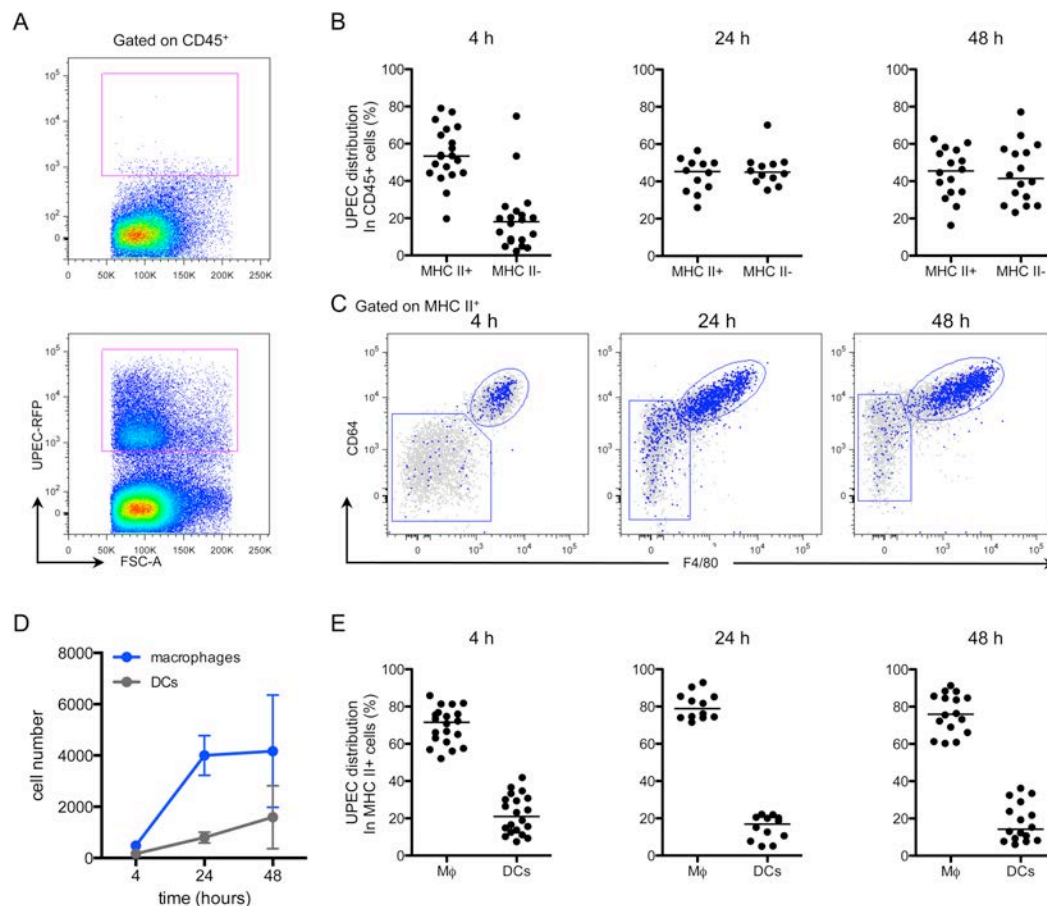


Figure 14: Among APCs, macrophages predominantly take up UPEC at early times post-infection. Female C57Bl/6 mice were instilled with UTI89-RFP and bladders were processed for flow cytometry at 4, 24, and 48 hours. (A) Representative plots, gated on CD45⁺ cells, depict the fluorescence level of UTI89-RFP in bladders, as compared to bladders infected with the nonfluorescent parental UTI89 strain. (B) Graphs depict the distribution of UPEC between MHC II⁺ and MHC II⁻ cell populations (gated first on CD45⁺ cells) over time. (C) Representative flow plots display the distribution of bacteria in the MHC II⁺ cell populations by overlay of bacteria⁺ cells (blue) on top of all CD45⁺ cells (gray). (D) Graph depicts the number of UPEC⁺ macrophages and DCs over time. (E) Graphs show the distribution of UPEC in CD45⁺ MHC II⁺ macrophages and DCs as a percentage of UPEC acquired by all MHC II⁺ cells. In (B) and (E), each dot represents one mouse, lines are medians. In (A) and (C), single representative bladders are depicted. Experiments were performed 4 times with 4-7 mice per group and results pooled in (B), (D), (E).

Infiltrating classical monocytes primarily become macrophages during infection

During the course of our infections, CD64 expression exhibited differential staining patterns within the macrophage gate (Figure 14C). As it has been reported that monocytes infiltrate the bladder during UPEC infection (Ingersoll et al., 2008), we hypothesized that CD64^{int} cells observed in the macrophage gate corresponded to monocyte-derived macrophages. Therefore, we investigated the fate of infiltrating monocytes. We used an *in vivo* labeling technique to monitor the trafficking and fate of classical and nonclassical monocytes into the bladder (Engel et al., 2008; Tacke et al., 2006). Using fluorescent nondegradable beads, classical or nonclassical monocytes can be labeled in circulation and then the tissue of interest is evaluated by flow cytometry to identify the presence of infiltrating monocytes. Mice were infected 24 hours after labeling either the classical or nonclassical monocytes in circulation and sacrificed at 4, 24 and 48 hours P.I. to analyze their bladders by flow cytometry. We observed a greater infiltration of classical monocytes than the nonclassical subset, which is in line with previous observations from other infection models (Ingersoll et al., 2011; Serbina and Pamer, 2006) (Figure 15A). As the majority of the infiltrating monocytes corresponded to classical monocytes, we focused our attention on this subset. We evaluated the expression of specific cell surface markers to define the phenotype of bead⁺ cells. From 24 to 48 hours P.I. CD11c, MHC II, and CD64 expression was upregulated in the majority of bead⁺ cells, while CD11b and Ly6C levels decreased (Figure 15B). Indeed, the percentage of bead⁺ cells with a monocytic phenotype decreased from 24 to 48 hours P.I. while bead⁺ cells with a macrophage phenotype increased (Figure 15C). Additionally, a smaller percentage, approximately 10% of all bead⁺ cells, had a DC phenotype. Taken together, these data show that upon infiltration into infected bladder, classical monocytes mainly differentiate to macrophages.

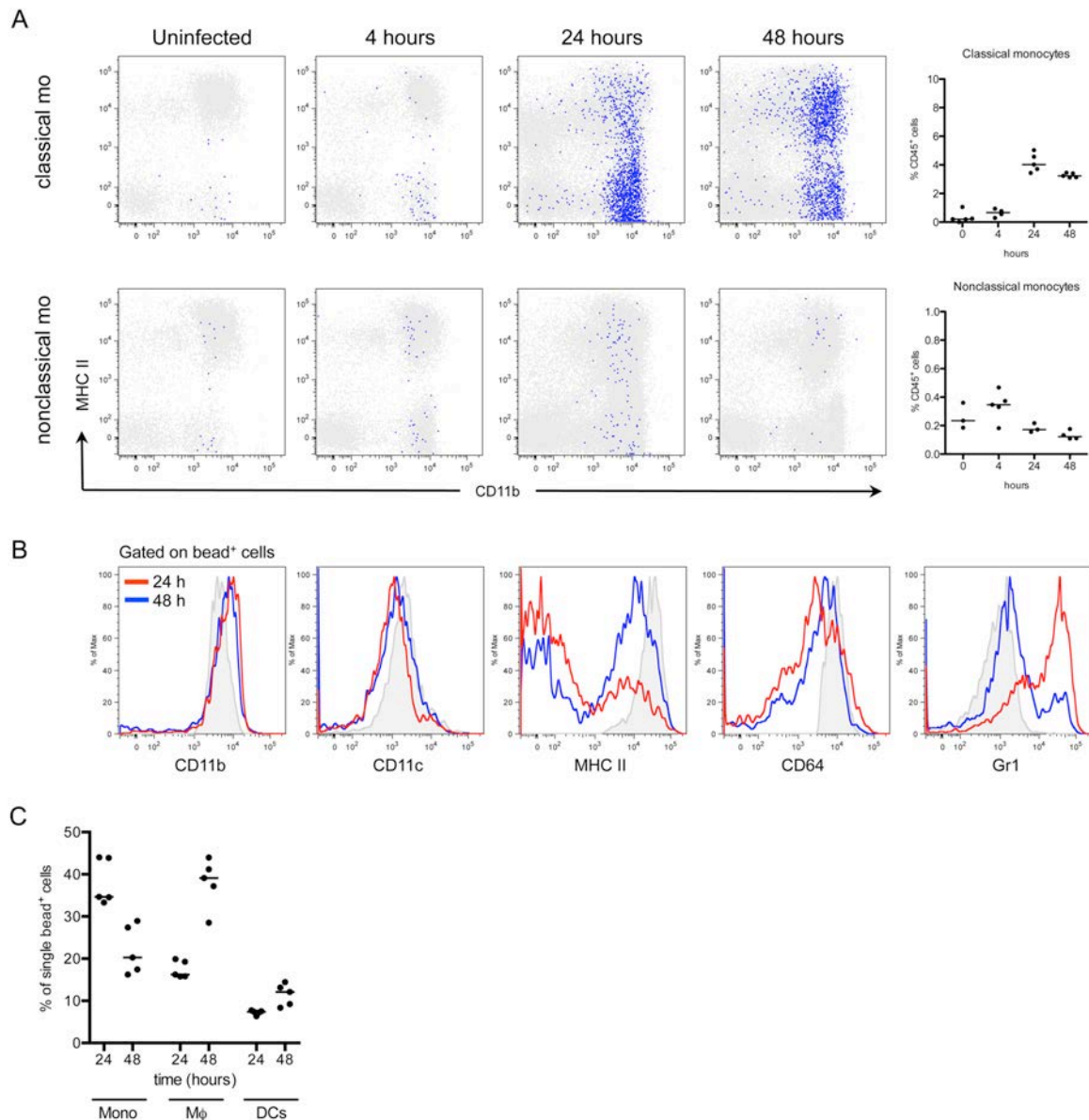


Figure 15: Classical monocytes robustly enter the bladder and become macrophages. In naïve C57Bl/6 mice, monocyte subsets were labeled *in vivo* as described in Materials and Methods. The designation “classical mo” and “nonclassical mo” indicates the monocyte subset labeled. Mice were infected with UTI89 24 hours after monocyte labeling and sacrificed at 4, 24, and 48 hours P.I. for flow cytometry. (A) Representative cytometry plots are shown, where gated bead⁺ cells (blue) are overlaid on all CD45⁺ bladder cells (gray). Graphs depict the quantitation of infiltrating monocytes over time, note difference in y-axis. (B) Histograms show the bead⁺ cell phenotype in the bladder over time after classical monocyte labeling (red lines - 24 hours P.I., blue lines - 48 hours P.I., and gray histograms - resident macrophages at 4 hours P.I., for reference). (C) Graphs show the percentage of all bead⁺ CD45⁺ cells after classical monocyte labeling in infected bladders by immune cell subset at 24 and 48 hours P.I. Each dot is one mouse, lines are medians. In (A) and (B), single representative bladders are displayed. Experiments were performed 3 times with 3-5 mice per group.

Bladder-resident macrophages negatively impact the induction of adaptive immunity to UPEC

Macrophages represent the majority of immune cells in the bladder and they are also the predominant APC acquiring UPEC early in infection. Additionally, during infection, infiltrating monocytes contributed to the macrophage compartment. Macrophages can influence the generation of an adaptive immune response through the modulation of the cytokine microenvironment (Schreiber et al., 2013; Silva, 2010) or through antigen sequestration (Kradin et al., 1999; MacLean et al., 1996). Thus we hypothesized that monocyte-derived and/or resident macrophages might impact the induction of adaptive immunity during UTI. To test this hypothesis, we depleted each population separately and evaluated the impact on bacterial clearance after primary and challenge infection. Circulating monocytes were depleted with clodronate liposomes (Van Rooijen and Sanders, 1994) (Figure 16A-B) and 15-18 hours later mice were infected with UPEC. Of note, clodronate treatment did not eliminate bladder-resident macrophages (Figure 16B). Mice were sacrificed 24 hours P.I. and bladder homogenates were plated to determine bacterial load. Monocyte-depleted animals had a small (<1-log) but significant improvement in bacterial clearance at 24 hours P.I. (Figure 17A). This difference, however, was lost if mice were infected 24 hours post-clodronate treatment, when monocytes have begun to repopulate the circulation, suggesting that monocyte depletion has a transient impact on bacterial burden. Supporting this conclusion, there were no differences in bacterial burden 24 hours P.I. in *CCR2*^{-/-} mice (Figure 18), which have greatly reduced, but not absent, numbers of circulating monocytes (Serbina and Pamer, 2006), as compared to WT mice.

Bladder-resident macrophages were eliminated by two consecutive injections of anti-CSF1R depleting antibody 24 hours prior to infection (Figure 16C) (Hashimoto et al., 2013). The anti-CSF1R antibody also targeted monocytes, however these cells were not completely eliminated at the moment of infection (Figure 16D). Macrophage-depleted and control mice had comparable bacterial burden at 24 hours P.I. (Figure 17B) suggesting that the absence of macrophages does not impact UPEC clearance at this time P.I.

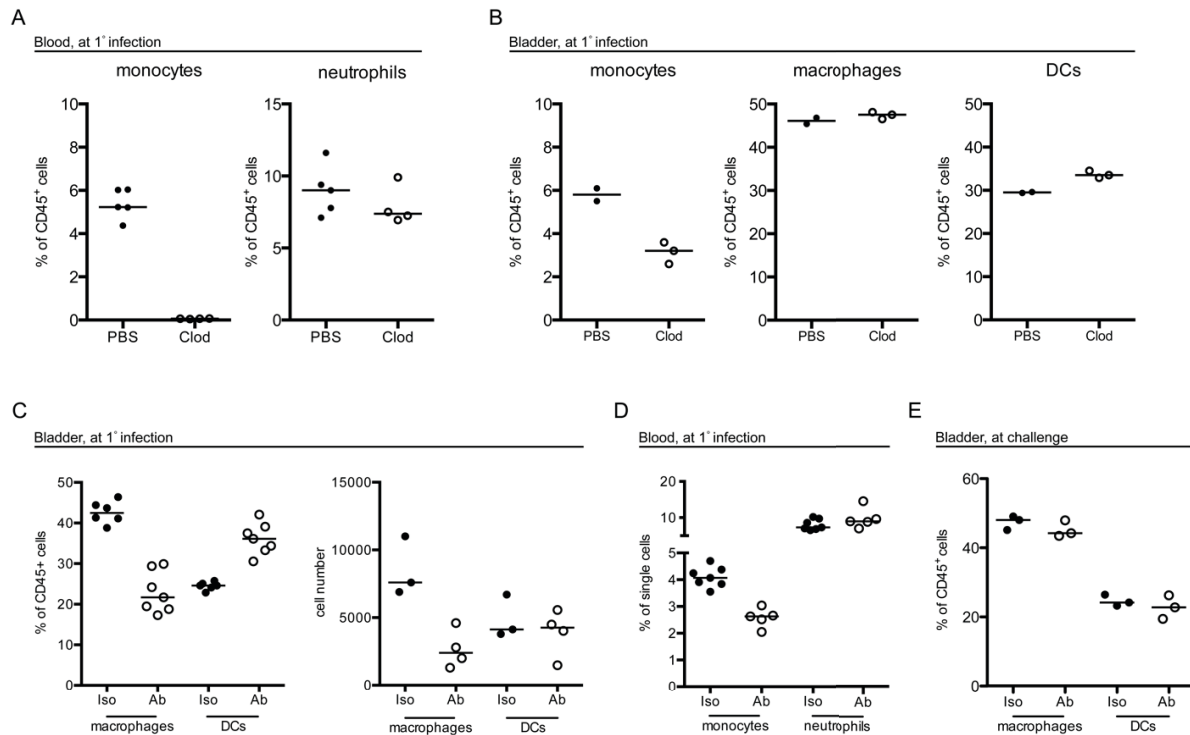
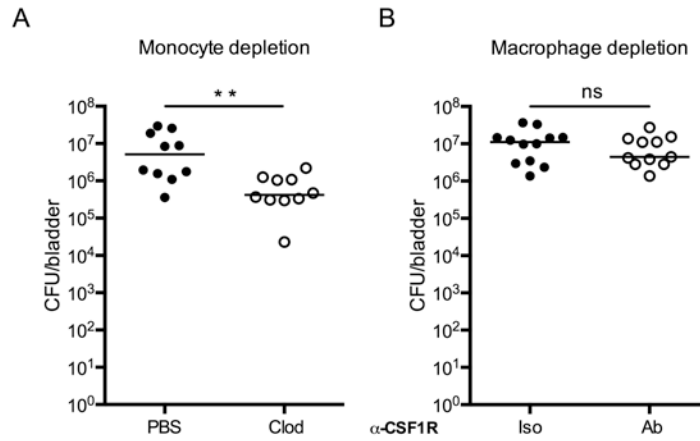


Figure 16: Immune cell ablation. (A-B) Mice were treated with PBS or clodronate liposomes (Clod) I.V. and 15-18 hours later, blood and bladder samples were obtained to evaluate immune cell depletion. Graphs depict the percentage of (A) monocytes and neutrophils in blood and (B) monocytes, macrophages, and DCs in the bladder after treatment. (C-D) Mice received two injections of anti-CSF1R antibody (Ab) or control isotype antibody (Iso) and 24 hours post-treatment, naive bladders were isolated to evaluate immune cell depletion. Graphs show the (C) percentage and cell number of macrophages and DCs in the bladder and (D) percentage of monocytes and neutrophils in the blood. (E) Mice were depleted of macrophages as in (C-D), however, bladders were evaluated for repopulation by macrophages 4 weeks after depletion, prior to challenge infection in additional cohorts of treated mice. Each dot represents one mouse. Experiments were repeated 2-4 times with 2-7 mice per group.

Primary infection



Challenge infection

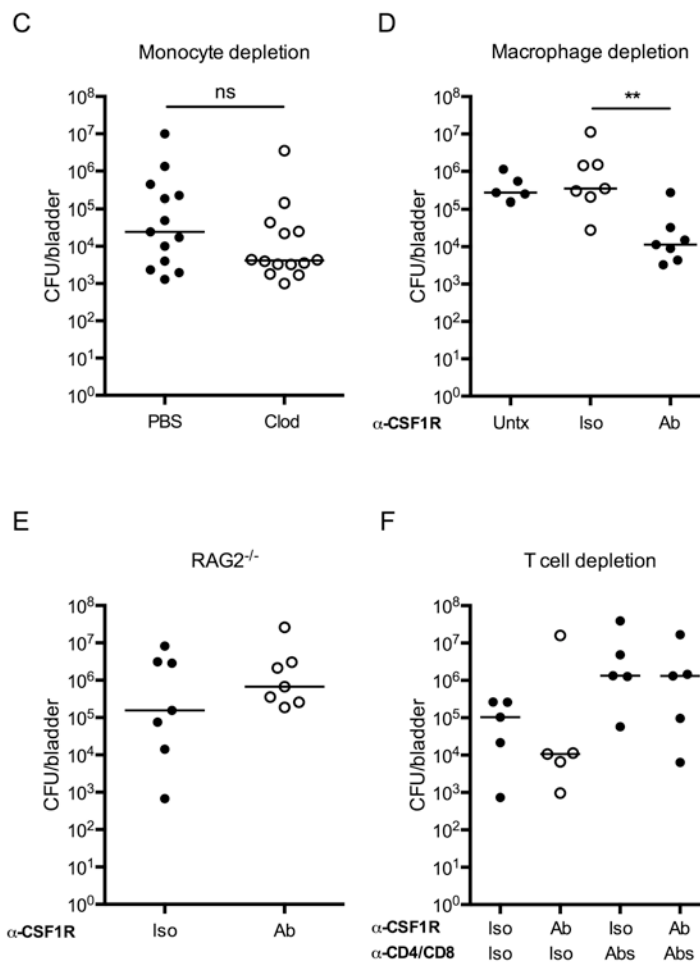


Figure 17: Macrophage depletion improves the adaptive response to UPEC infection.

Graphs show CFU per bladder 24 hours post primary infection (A) in mice treated with PBS or clodronate liposomes (Clod) to deplete monocytes 15-18 hours prior to infection and (B) in mice treated 24 hours prior to primary infection with isotype control (Iso) or CSF1R antibody (Ab). (C-F) Mice were given a primary infection, allowed to resolve, and 3 to 4 weeks P.I.,

mice were challenged with an isogenic UPEC strain, as in Fig. 1A. Graphs show CFU per bladder 24 hours post challenge infection in (C) wildtype mice treated with PBS or clodronate-loaded liposomes (Clod) to deplete monocytes 15-18 hours prior to primary infection, (D) isotype (Iso) or CSF1R antibody (Ab) treated mice to deplete resident macrophages 24 hours prior to primary infection, or left untreated (Untx), (E) RAG2^{-/-} mice treated with isotype (Iso) or CSF1R antibody (Ab) prior to primary infection or, (F) wildtype mice treated with isotype (Iso) or CSF1R antibody (Ab) to deplete resident macrophages and isotype (Iso) or anti-CD4 and anti-CD8 antibodies (Abs) to deplete T cells, prior to primary infection. Each dot is one mouse, lines are medians. Experiments were performed 2-4 times with 4-7 mice per group. **p=0.0021, Mann-Whitney; ns: not significant.

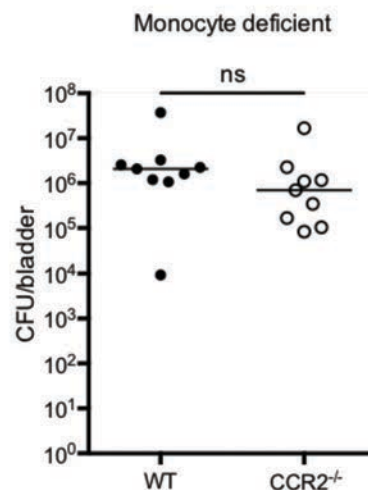


Fig 18: CCR2^{-/-} mice are not impaired in bacterial clearance after primary infection. Graph depicts the CFU/bladder 24 hours post-primary infection in wildtype (WT) or CCR2^{-/-} mice. Experiment was repeated 2 times with 4-5 mice per group.

We then evaluated the role of monocytes and macrophages in the generation of an adaptive immune response. Indeed, macrophages can impact adaptive immunity via cytokine secretion or antigen sequestration (Kradin et al., 1999; Schreiber et al., 2013). To directly evaluate the influence of these cells on the induction of adaptive immunity during UTI, we depleted each population separately, as above, prior to primary infection. Monocytes were depleted by clodronate treatment and 15-18 hours post-depletion mice were infected, allowed to clear their infection, and then challenged with an isogenic UTI89 strain. Twenty-four hours post-challenge mice were sacrificed to determine bacteria titers. We did not observe differences in

bacterial burden post-challenge (Figure 17C) or in reservoir formation (Figure 19A), suggesting that the absence of monocytes at the moment of the primary infection did not influence the response to UPEC challenge.

To determine if resident macrophages impact bacterial clearance after challenge, we treated mice with anti-CSF1R as above and infected with UPEC. Once the primary infection was cleared, mice were challenged with an isogenic UTI89 strain and CFU were measured at 24 hours post-challenge. While macrophage absence did not impact UPEC clearance during a primary infection (Figure 17B), we observed a surprising reduction of nearly 2 orders of magnitude in CFU after challenge infection in macrophage-depleted mice compared to control isotype-treated and untreated (infected and challenged but not receiving any antibody injection) mice (Figure 17D). Importantly, at the time of challenge infection, macrophages had repopulated the bladder in depleted animals (Figure 16E), discarding the possibility that reduced bacterial clearance during challenge was directly due to the absence of macrophages. Similar to that observed in the $RAG2^{-/-}$ or CD11c-depleted mice, reservoir formation was not impacted by macrophage depletion (Figure 19B).

To evaluate if this improvement in bacterial clearance in the absence of macrophages was dependent on the adaptive immune system, we depleted macrophages and performed the same experiment as above in $RAG2^{-/-}$ mice. We observed no difference in bacterial clearance following challenge infection between macrophage-depleted or control treated $RAG2^{-/-}$ mice (Figure 17E). To specifically address the necessity of T cells, we depleted macrophages in mice depleted or not of T cells. To deplete T cells, mice were treated with anti-CD4 and CD8 depleting antibodies prior to primary infection and at 5 days intervals throughout infection. We observed an improvement in bacterial clearance in the absence of macrophages only in mice with an intact T cell compartment. Mice depleted of $CD4^{+}$ and $CD8^{+}$ T cells had similar bacterial burden independently of macrophage depletion (Figure 17F). Bacteria titers in T cell depleted mice were similar to titers observed during a primary infection, supporting our findings in $RAG2^{-/-}$ mice, and demonstrating that T cells are necessary to mount an adaptive immune response against UPEC. Taken together, these results support the conclusion that macrophage depletion at the prior to primary infection positively impacts the generation of an adaptive immune response against UPEC.

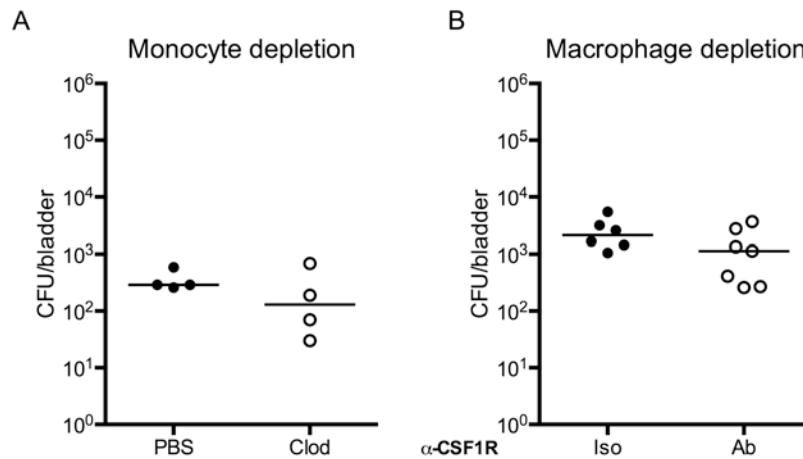


Figure 19: UPEC reservoirs are not altered in monocyte or macrophage depleted mice. Graphs depict CFU/bladder arising from the primary infecting strain in an experiment in which (A) monocytes or (B) macrophages were depleted prior to primary infection and then challenged with an isogenic strain and sacrificed 24 hours post-challenge. Each dot represents one mouse. Experiments were repeated 2-4 times with 2-7 mice per group.

Effector cell infiltration is unchanged during UPEC challenge among macrophage-depleted and control treated mice

To investigate potential mechanisms mediating improved clearance after macrophage depletion in a challenge infection, we evaluated the infiltration of effector cells during UPEC challenge in macrophage-depleted and control animals. We depleted macrophages and infected the mice as in Figure 10. Upon resolution of the primary infection, mice were challenged and sacrificed 24 hours P.I. for analysis by flow cytometry. We evaluated the infiltration of T and B cells, as well as neutrophils and monocytes into the bladder. Surprisingly, we did not observe differences in the number of T or B cells infiltrating the tissue (Figure 20A) nor any difference in the number of neutrophils or monocytes (Figure 20B). These results suggest that improved bacterial clearance infection is not mediated by increased numbers of innate or effector cells infiltrating the bladder during challenge.

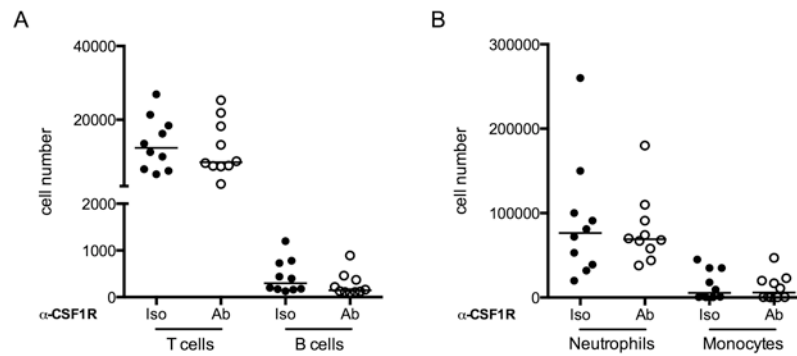


Figure 20: Macrophage depletion does not impact effector cell infiltration during UPEC challenge. (A-B) Female C57Bl/6 mice were infected and then challenged with UPEC after resolution of their primary infection, as in figure 10. Twenty-four hours post-challenge, cellular infiltration into the bladder was evaluated by flow cytometry. Graphs depict cell number per bladder of the indicated populations. Experiment was repeated 3 times with 5-7 mice per group.

UPEC-specific antibodies are undetectable during infection

In addition to cell infiltration, we evaluated UPEC-specific antibodies as a measure of the adaptive immune response generated during primary infection in macrophage-depleted and control treated mice. Urine samples were collected during the course of different experiments to evaluate UPEC-specific IgA by ELISA. We observed that UPEC-specific IgA was at the limit of detection of the assay and never significantly higher than the levels detected in uninfected mice (Figure 21A) and for this reason we could not draw any conclusions regarding the generation of UPEC-specific antibodies in macrophage-depleted and control mice. As an alternative approach, we evaluated UPEC-specific IgA in bladder lysates, however the levels were also at the limit of detection of the assay (Figure 21B). Of note, it has been reported that UPEC-specific antibodies are not generated during bladder infection (Chan et al., 2013 ; Ratner et al., 1981).

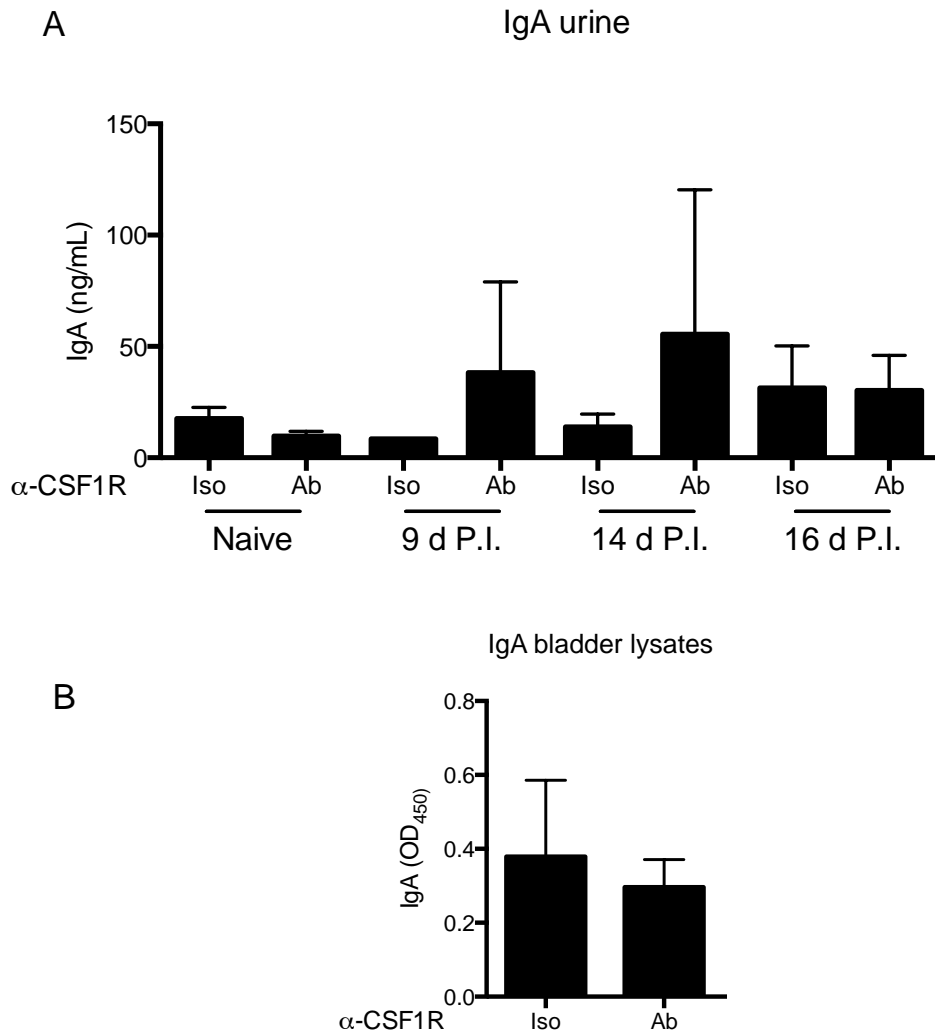


Figure 21: UPEC-specific IgA remained at the limit of the detection. (A-B) Female C57Bl/6 mice were depleted or not of macrophages and infected 24 hours post-depletion with 10^7 CFU of UPEC. (A) Urine was collected during the course of the infection and IgA was evaluated at different days (d) post-infection (P.I.). (B) Once the primary infection was resolved, mice were challenged with UPEC. Twenty-four hours post-challenge, bladders were isolated and homogenized. UPEC-specific IgA in bladder homogenates was evaluated by ELISA. Graph depicts the mean of IgA levels in isotype (Iso) and antibody (Ab) treated mice. Experiment was repeated 2 times with 5-6 mice per group.

Macrophage depletion does not change the cytokine microenvironment in the bladder during primary infection

Macrophages can influence adaptive immunity by modulating the cytokine microenvironment (Schreiber et al., 2013; Silva, 2010). Therefore, we treated the mice with macrophage depleting or control antibodies and evaluated cytokine expression in the bladder 24 hours after a primary infection by luminex technology. Notably, we did not observe significant differences between macrophage-depleted and control mice in any of the 32 cytokines evaluated (Figure 22), suggesting that cytokine expression is not significantly impacted by macrophage absence at 24 hours P.I.

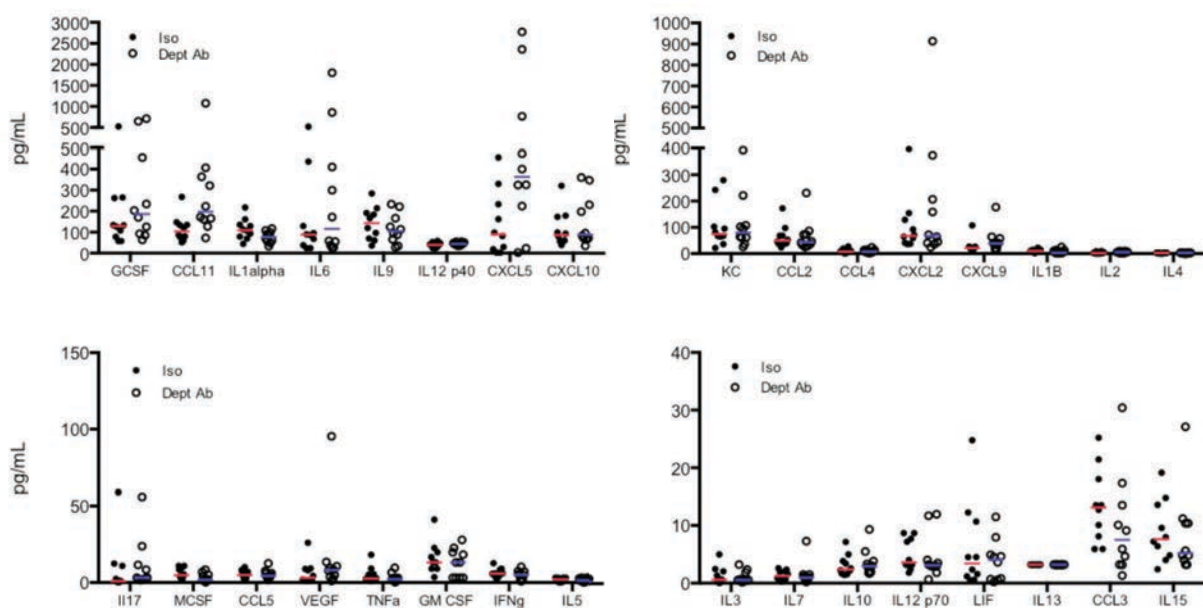


Figure 22: Macrophage depletion does not impact cytokine expression in the bladder. Mice were depleted with anti-CSF1R antibody and infected with 1×10^7 CFU of UTI89 24 hours after depletion. Mice were sacrificed 24 hours P.I. and bladders were homogenized. Samples were stored at -80°C until all samples could be assessed together by Luminex multi-analyte profiling. Graphs depict the expression levels the evaluated cytokines in isotype antibody treated (black dots) and depleting-antibody treated (open circles) mice. Analytes are grouped by high expression (top graphs) and low or no expression (bottom graphs). Each dot represents a mouse, experiment performed 2 times with 5 mice per group and pooled, lines are medians.

Increasing the amount of antigen does not affect the generation of adaptive immunity during UTI

In the lung, it has been observed that macrophages can influence the generation of an adaptive immune response by antigen sequestration, hindering antigen presentation and subsequent T cell priming by DCs (Jakubzick et al., 2008; Jakubzick et al., 2006; Kradin et al., 1999; MacLean et al., 1996). It has been proposed that DC migration and induction of adaptive immunity can only occur in the lung after macrophages have been saturated and there is an excess of antigen available to the DCs (Kradin et al., 1999; MacLean et al., 1996). To test the hypothesis that antigen availability during primary infection impacts the generation of an adaptive immune response during UTI, we infected untreated mice with 10^7 , 10^8 , or 10^9 CFU of UPEC. Once the primary infection was resolved, mice were challenged with 10^7 CFU of an isogenic UPEC strain and 24 hours P.I., sacrificed to determine bacterial burden. We did not observe a difference in the number of bacteria per bladder after a primary infection despite increasing the inoculum 100-fold (Figure 23A). Furthermore, we did not observe any differences in bacterial load after challenge among the three groups (Figure 23B). These data suggest that increasing the number of bacteria during primary infection does not improve the generation of adaptive immunity during UTI, however it is possible that we did not saturate bladder resident macrophages with bacteria and that it is necessary to increase the number of bacteria used for infection. We did not check if the number of DCs acquiring UPEC among the different groups (mice infected with 10^7 , 10^8 , or 10^9 CFU of UPEC) increased, therefore an alternative explanation for this result could be that independently of the number of bacteria used to infect the mice, the amount of DCs harboring UPEC was the same.

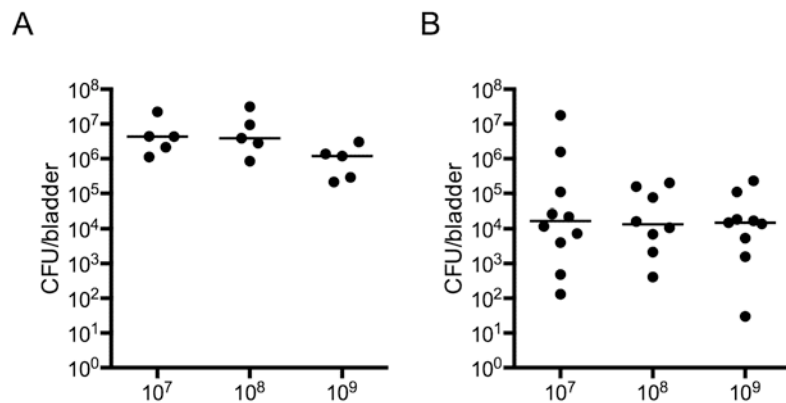


Figure 23: Increasing bacterial inoculum during primary infection does not improve the response to challenge infection. Female C57Bl/6 mice were instilled with 1×10^7 , 1×10^8 , or 1×10^9 CFU of UPEC and challenged with 1×10^7 CFU after resolution of the primary infection as in figure 10. (A) Plot depicts CFU at 24 hours post-primary infection. (B) Graph shows CFU 24 hours post-challenge. Each dot represents one mouse. Experiments were repeated 2 times with 5-7 mice per group.

Macrophage depletion leads to increased phagocytosis of UPEC by DCs

As the bladder microenvironment was unchanged at 24 hours P.I. in the absence of macrophages, we evaluated whether macrophages were physically sequestering UPEC in the bladder during UTI by measuring bacterial acquisition in the bladder in the context of macrophage depletion. Mice were depleted or not of macrophages by anti-CSF1R antibody treatment, infected with UTI89-RFP, and sacrificed 24 hours P.I. We then evaluated the immune cells containing UPEC (RFP⁺) by flow cytometry as in Figure 13. The distribution of UPEC was altered in mice depleted of macrophages as compared to the control group. A greater percentage of MHC II⁺ cells contained UPEC compared to MHC II⁺ cells (Figure 24A). Additionally, the percentage of neutrophils containing bacteria increased in macrophage-depleted mice, likely compensating for the lack of monocytes and resident macrophages (Figure 24B). In MHC II⁺ cell populations, while the total number of DCs in infected bladders was unchanged (Figure 24C), we observed that a significantly greater percentage of DCs had taken up UPEC in macrophage-depleted animals (Figure 24D). Moreover, the percentage of UPEC-containing resident macrophages and monocyte-derived macrophages remained unchanged (Figure 24E). These results suggest that macrophages sequester UPEC from DCs and in their absence, more DCs are now able to acquire UPEC positively impacting the induction of an adaptive immune response.

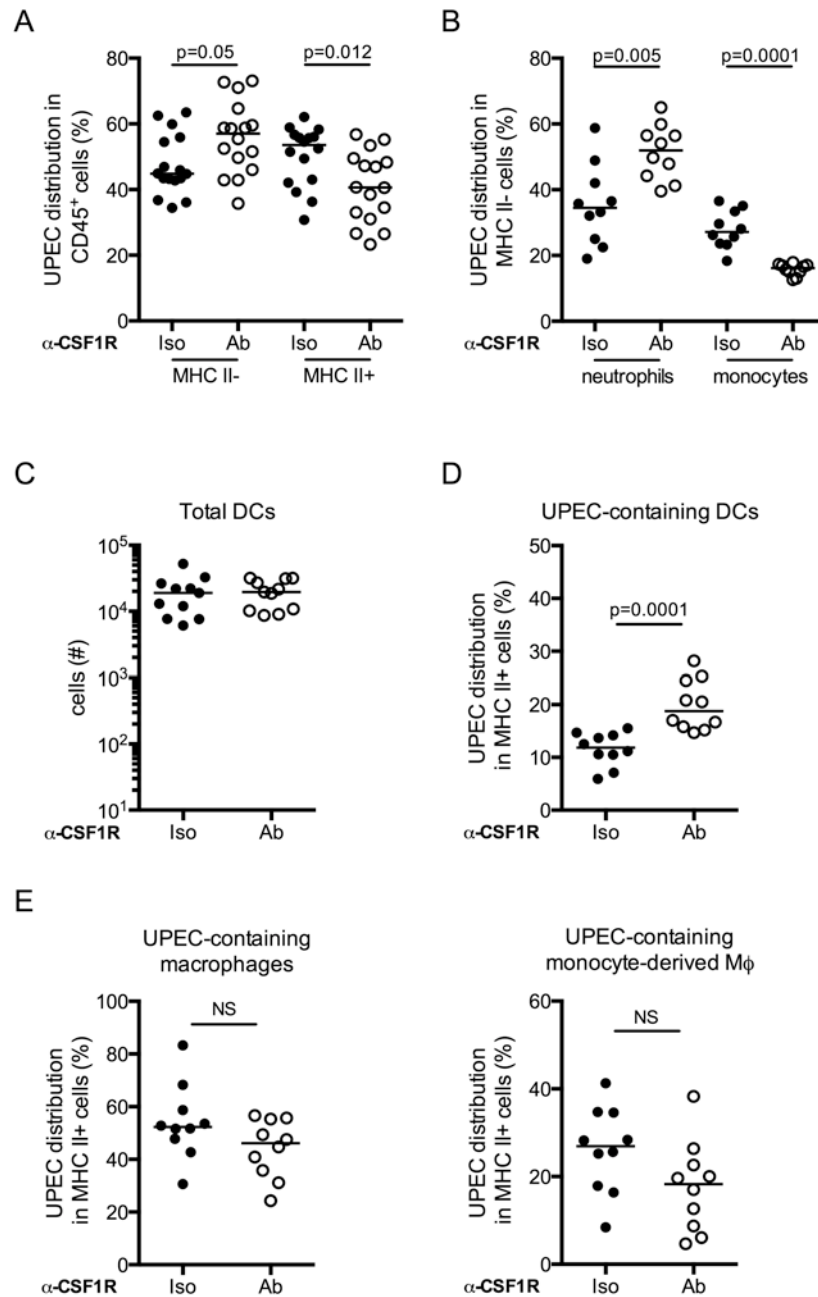


Figure 24: DCs acquire more bacteria in the absence of macrophages. Macrophages were depleted or not and mice were infected. Twenty-four hours post-primary infection, bladders were analyzed by flow cytometry to determine distribution of UPEC in (A) the MHC II⁻ and MHC II⁺ compartments, (B) neutrophils and monocytes. (C) Plot depicts the total number of DCs present in the bladder 24 hours post-primary infection. (D-E) Plots depict the percentage distribution of UPEC in the MHC II⁺ compartment by cell population (D) DCs and (E) resident macrophages and monocyte-derived macrophages. Each dot represents one mouse, lines are medians. Experiments were repeated 3 times with 4-7 mice per group in each experiment and results pooled. p-values indicated on graphs, Mann-Whitney test.

Chapter 3: Delineating the adaptive immune response generated during UTI

Introduction

The results presented in this chapter are part of our ongoing efforts to further delineate the adaptive immune response to UTI. We are currently focusing on unraveling the role of the T cell. The unpublished work presented here represents our first findings.

We found that the adaptive immune system was necessary for improved bacterial clearance after UPEC challenge. The adaptive immune responses generated against pathogens can be classified as cellular or humoral, depending whether they are mediated by T or B cells, respectively. In general, intracellular pathogens, such as viruses and intracellular bacteria, trigger a cellular response that includes the generation of specific CD8⁺ T cells, which are necessary to detect, and clear infected cells. On the other hand, extracellular pathogens commonly induce an antibody-mediated response. Of note, both responses can be generated during infection; as an example, HIV infection induces virus-specific T cells as well as virus-specific antibodies (Xu et al., 2013). It is not completely clear whether a cellular response alone or a humoral response alone is able to control UPEC infection during challenge or if both responses are needed. Therefore, we focused our attention on dissecting the adaptive immune response generated during UTI, considering that broadening our knowledge is key to the development of future therapeutic options.

DCs mediate communication between the innate and adaptive immune response. They phagocytize pathogens and infected cells, sense the microenvironment, and migrate to LNs to deliver information to naïve lymphocytes. Depending on their origin, function, and distribution, DCs can be classified into different subsets including CD4⁺ and CD8α⁺ DCs (Kushwah and Hu, 2011). CD103⁺ DCs, which are particularly important to induce a CD8⁺ T cell response, reside in nonlymphoid tissues and cross-present antigens (Bedoui et al., 2009; del Rio et al., 2010; Ginhoux et al., 2009). The transcription factor Batf3 has been shown to be fundamental for the development of CD8α⁺ DCs (LN-resident DCs) and CD103⁺ DCs, as mice lacking this protein also lack both of these DC subsets and have an impaired CD8⁺ T cell response (Hildner et al., 2008). Over the course of our studies, we reported that DCs and T and B cells are necessary to reduce CFU after UPEC challenge (Mora-Bau et al., 2015), however, which DC subset is involved in the induction of adaptive immunity during UTI or what type of T cells are generated during this response is unknown. We observed that CD103⁺ DCs reside in the bladder mucosa, and thus evaluated the role of these DCs during UTI.

CD8 α ⁺ and CD103⁺ DCs are dispensable for an adaptive immune response against UPEC

CD8 α ⁺ DCs and CD103⁺ DCs are more efficient cross-presenting cells and therefore fundamental for the induction of CD8⁺ T cell responses (Bedoui et al., 2009; Dudziak et al., 2007; Hildner et al., 2008). The role of CD8⁺ T cells during UTI has not been addressed before. This is surprising considering that UPEC invades uroepithelial cells and a CD8⁺ T cell response would be beneficial to fight intracellular bacteria. To evaluate the role of CD8 α ⁺ DCs and CD103⁺ DCs during UTI, we utilized Batf3 KO mice. Batf3 KO and WT mice were infected with 10⁷ CFU of UPEC and their urine was monitored every 5-6 days to evaluate the presence of bacteria. When no bacteria in the urine were detected, mice received a challenge infection with an isogenic UPEC strain as in Figure 10. We did not observe any difference in bacterial clearance between Batf3 KO and WT mice suggesting that CD8 α ⁺ DCs and CD103⁺ DCs are not necessary to induce an adaptive immune response against UPEC (Figure 25A). We did not observe any difference in the reservoir either (Figure 25B). As CD8 α ⁺ DCs and CD103⁺ DCs are thought to be essential to induce cytotoxic T cells, CD8⁺ T cell responses should be absent in Batf3 KO mice. The use of these mice can be considered to be an indirect means to evaluate CD8⁺ T cell responses. With this in mind, our results suggest that a CD8⁺ T cell response is dispensable for UPEC clearance during challenge infection. The lack of a CD8 T cell response may explain why bacterial reservoirs persist, even in the face of a challenge infection. It has been reported, however, that CD8⁺ T cell responses can arise in Batf3 KO mice, as CD8 α ⁺ DCs are induced in the absence of the Batf3 transcription factor and cross-present soluble antigens and cell-associated antigens during GVHD (Seillet et al., 2013).

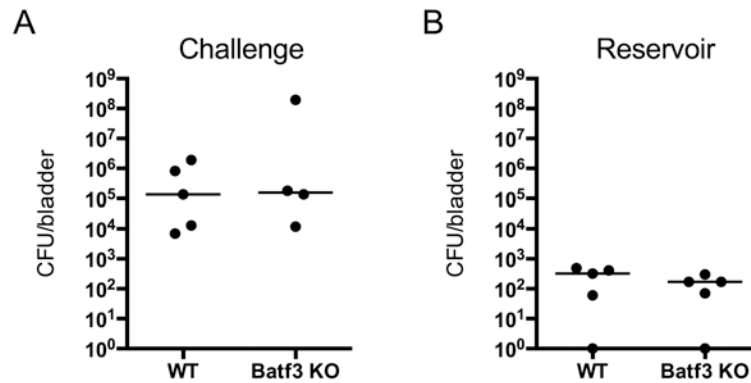


Figure 25: The absence of CD8 α ⁺ and CD103⁺ DCs does not impact the adaptive immune response against UPEC. WT and Batf3 KO mice were infected with 10⁷ CFU of UPEC. Once the mice resolved the primary infection, they were challenged with an isogenic strain of UPEC. Twenty-four hours post-challenge, mice were sacrificed and bacterial load was evaluated. Graphs depict bacterial load derived from the challenge (A) and primary infecting strains (bacteria reservoirs) (B). Experiment was repeated 3 times with 5-6 mice per group.

T cells are dispensable for the direct response to UPEC during challenge infection

In previous experiments, we observed that a lack of T cells over the course of primary infection negatively impacted bacterial clearance during UPEC challenge (Fig 17F). However, this experimental approach did not allow us to determine whether T cells are necessary to directly kill bacteria during challenge infection or if they are required to generate a humoral response against UPEC. To directly test whether T cells, and in particular CD8⁺ T cells, were necessary as part of the challenge response, we modified our T cell depletion experiment. We depleted T cells one day prior to challenge infection, instead of prior to primary infection, a strategy that would permit the induction of T cell immunity. Mice were infected with 10⁷ CFU of UPEC and urine was monitored every 5-6 days to evaluate the resolution of primary infection. When the acute infection was resolved, mice were treated with anti-CD4 and CD8 depleting antibodies or isotype controls 24 hours prior to challenge infection. Twenty-four hours post-challenge, mice were sacrificed and bacterial burden was evaluated. We observed no difference in bacterial clearance between T cell depleted and

control isotype treated mice, suggesting that T cells are not necessary to clear UPEC during challenge infection (Figure 26).

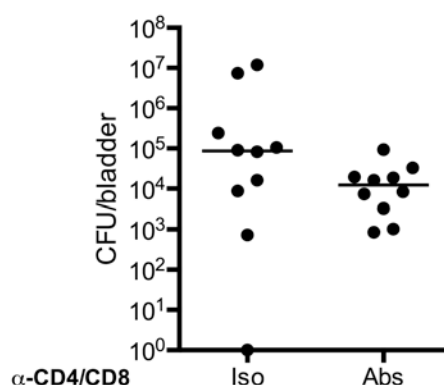


Figure 26: T cells are not necessary for UPEC clearance during challenge infection. Female C57Bl/6 mice were infected with 10⁷ CFU of UPEC. Once mice had resolved the primary infection, CD4⁺ and CD8⁺ T cells were depleted by administration of anti-CD4 and anti-CD8 depleting antibodies. Twenty-four hours post-depletion, mice were challenged with an isogenic strain of UPEC and 24 hours later sacrificed to evaluate bacterial load. Experiment was repeated 3 times with 5-6 mice per group.

In summary, the results presented here, suggest that T cells are not necessary to directly clear UPEC during challenge infection and/or to clear bacterial reservoirs. However, the response during a challenge infection is impaired when T cells were depleted at the prior to infection (Figure 17F) suggesting a role for these cells in the development of an adaptive immune response during UTI. We hypothesize that T cells are required for an antibody-mediated response against UPEC and that these antibodies are sufficient to clear UPEC during a challenge infection. It will be interesting to continue studying the role of T and B cells during UTI to answer questions such as: 1) what kind of T cells are generated during UTI? 2) Are UPEC-specific CD8⁺ T cells generated during UTI? If so, 3) are they truly dispensable during infection? 4) Can we improve the antibody response generated during UTI to improve bacterial clearance? 5) Can we induce or augment the CD8⁺ T cell response to eliminate UPEC reservoirs? To answer these questions, we are in critical need of specific tools to be able to identify UPEC-specific lymphocytes, including UPEC-specific MHC tetramers. To facilitate our search for specific lymphocytes, we have recently engineered UPEC strains to

express OVA peptides, which can be recognized by transgenic CD4⁺ and CD8⁺ T cells (T cells from OT II and OT I mice) and an altered flagellin, which can be recognized by transgenic CD4⁺ T cells (T cells from SM1 mice). With these tools, we will be able to evaluate if UPEC-specific T cells responses are generated and further characterize this response to achieve greater understanding of the adaptive immune response occurring during UTI to provide better solutions to treat UTI patients.

Chapter 4: Discussion

Portions of the manuscript “Macrophages Subvert Adaptive Immunity to Urinary Tract Infection” published in Plos Pathogens (Mora-Bau et al., 2015) have been reproduced here.

UTI is unusual in that it is a common infection, affecting otherwise healthy individuals, that recurs with high frequency, suggesting a defect in the ability to build an adaptive immune response to UPEC. We observed that the absence of B and/or T cells or DCs impaired the host’s capacity to clear bacteria after a challenge infection, confirming that adaptive immune responses are primed and necessary during UTI. Although this was an expected result, it has never been formally demonstrated before. Notably, while we found that an adaptive immune response is primed, and bacterial clearance after a challenge infection is impaired in the absence of DCs, B and T cells, intact mice were not fully protected from challenge infection. To shed light on potential mechanisms preventing the development of a more protective adaptive immune response to UPEC infection, we focused on the role of MHC II⁺ cells, as they are the central initiators of immunity. In the course of our work, our key finding was that, in the context of a challenge infection, resident macrophage depletion improved the host’s ability to eliminate bacterial load. Importantly, macrophages were depleted prior to the first infection; however, they were present in normal numbers at the time of challenge infection indicating that it was not the absence of macrophages during the second infection that impacted bacterial clearance during challenge. This improvement was dependent on the adaptive immune system as the phenotype was lost when macrophages were depleted in RAG2^{-/-} mice or in mice depleted of T cells. Given that macrophages were the principal APC to acquire UPEC early in infection, these data suggest a model in which macrophages sequester the bacteria from the DCs subverting the initiation of a robust adaptive immune response during UTI.

How do macrophages limit the adaptive immune response against UPEC?

To understand how macrophages limit induction of an adaptive immune response during UTI, we evaluated events post-challenge and post-primary infection. We were specifically interested in answering two fundamental questions: (1) which cells are responsible for improved clearance during bacterial challenge in macrophage-depleted mice and (2) what happens in the bladder during primary infection, in the absence of macrophages,

that improves adaptive immunity against UPEC? To address the first question, we evaluated immune cell infiltration into the bladder during challenge infection. We observed a significant infiltration of T cells and a smaller infiltration of B cells post-challenge but no major differences in cell numbers between the control and treated groups. At this time, we cannot rule out potential qualitative differences in the activation or specificity of the infiltrating effector cells, and this is an active line of investigation. To gain more knowledge in this regard, it would be ideal to characterize what kind of T cells are infiltrating the bladder during challenge infection (*e.g.*, T helper bias, regulatory T cells) and determine if these T cells are different between macrophage-depleted and control-treated mice (*e.g.*, activation status, cytotoxic potential).

To answer the second question, we evaluated events occurring in the bladder in the first hours following primary infection, in the absence or presence of macrophages, which may impact the generation of an adaptive immune response. It has been shown that macrophages can influence the tissue microenvironment and in this way, modulate the induction of adaptive immunity (Schreiber et al., 2013; Silva, 2010). For example, during infection with *Citrobacter rodentium*, macrophages induce T_H1 polarization of CD4 T cells by secreting IL-12 (Schreiber et al., 2013). Furthermore, a recent study suggested that IL-10 expression from mast cells suppresses adaptive immunity to UPEC (Chan et al., 2013). Thus, hypothesizing that the absence of macrophages might influence the microenvironment, either directly or indirectly, we evaluated cytokine production in the bladder during infection in macrophage-depleted and control-treated mice. Multi-analyte cytokine analysis revealed no striking differences between control and depleted mice and in contrast to the study mentioned above, we did not detect IL-10 expression. The reasons for the different finding in our study and Chan et al. study are unclear. In the course of our study, we found few mast cells in naive bladder tissue. In addition, the differences may be due to the significant variation that exists in the genomes of commonly used strains such as cystitis strain UTI89, pyelonephritis strains J96, 563, CFT073, and clinical isolates (see phylogenetic tree in (Petty et al., 2014)). Together, our results suggest that macrophage absence does not modulate the bladder microenvironment during UTI, however, as we only evaluated cytokine expression at 24 hours P.I., we cannot rule out the possibility that the microenvironment was altered at a different time point.

Additionally, it has been observed that macrophages can limit the adaptive immune response by antigen sequestration (McLean et al. 1996; Kradin et al. 1999; Jakubzick et al., 2006). For example, after injecting mice intratracheally with heat-killed *Listeria*, macrophages acquire the majority of the antigen, limiting heat-killed *Listeria* acquisition by DCs; therefore, an adaptive immune response is achieved only after macrophage depletion (MacLean et al., 1996). Jakubzick and colleagues found that DC migration and antigen presentation only occurred when alveolar macrophages were over-saturated with antigen, allowing DC antigen uptake to occur. To explore the hypothesis of antigen sequestration during UTI, we evaluated which immune cell populations acquired UPEC in the bladder in the absence of macrophages. We observed an increase in the percentage of DCs containing bacteria in macrophage-depleted mice compared to control-treated animals. DCs are key players in initiating adaptive immune responses, and we found that they can do so from the bladder mucosa in the context of UTI. Indeed, even a partial depletion of bladder-resident DCs, prior to primary infection, rendered animals less capable of clearing bacteria after challenge infection. The intermediate clearance phenotype observed in DT-treated mice may have been mediated by DCs remaining after depletion or by DCs repopulating the tissues before the primary infection was resolved, permitting delayed antigen presentation; this suggests that a small number of DCs is sufficient to mount an adaptive immune response against UPEC. Thus, our data suggest that the more efficient adaptive immune response against UPEC observed during macrophage depletion may be mediated by the increase in the percentage and number of DCs carrying bacteria. If DCs in the bladder have limited access to UPEC due to macrophage sequestration, we could hypothesize that depleting neutrophils, which also acquire a significant number of bacteria during infection (see MHC II⁺ cells containing UPEC in Figure 14) (Mora-Bau et al., 2015), would also increase UPEC availability for DCs and improve the adaptive immune response in UTI. However, we would have to consider that in the absence of neutrophils, mice display improved bacterial clearance during primary infection (Ingersoll et al., 2008), which could affect the induction of adaptive immunity.

While depletion of bladder resident macrophages impacted the outcome in UPEC infection, monocyte depletion had no effect. The majority of monocytes infiltrating the bladder gave rise to macrophages, however some of these cells also became DCs. Therefore, monocyte depletion impacted the number of macrophages in the bladder but it also impacted the number of DCs and this may be the reason why the adaptive immune response against

UPEC was not improved in monocyte-depleted mice. We might imagine that a decrease in DC number would negatively impact the generation of an adaptive immune response against UPEC, however, as the total number of macrophages was also decreased as an indirect effect of clodronate treatment, remaining DCs may have had more access to UPEC. This hypothesis could be tested in a rigorous manner, by monocyte depletion and quantification of the total number of DCs as well as the number of DCs carrying bacteria, over several timepoints.

It is possible that macrophages are the main APC in the bladder to acquire UPEC because they outnumber DCs in both naïve and infected bladders. Alternatively, it is possible that the localization of macrophages and DCs in the bladder impact phagocytosis. If macrophages are positioned closer to the bladder lumen, they will have a greater sampling capacity during infection. To shed some light onto these questions, we are currently performing *ex vivo* experiments to evaluate UPEC acquisition by DCs and macrophages. By varying the proportion of these two cell types in the cultures, we can determine whether, for example, when we have the same number of DCs and macrophages then both cell types can acquire UPEC equally or there is still a bias in phagocytosis. Additionally, in this set-up, we have removed differences in cellular localization. To more directly address the question of localization, we will image the bladder.

Having defined an early role for resident macrophages and DCs during UTI (Figure 27), our work significantly advances the understanding of how adaptive responses to UPEC are achieved. Our data point to a barrier in the immune system that must be overcome, particularly for patients with recurrent UTI. Although macrophage sequestration of particulate antigen in the lung has been described (Jakubzick et al., 2006; Kradin et al., 1999; MacLean et al., 1996), this is, to the best of our knowledge, the first study to propose a role for the physical sequestration of antigen during live bacterial infection. Strategies that increase DC number or DC migration may overcome the subversion imposed by macrophages, providing a viable solution to treat patients with recurrent UTI.

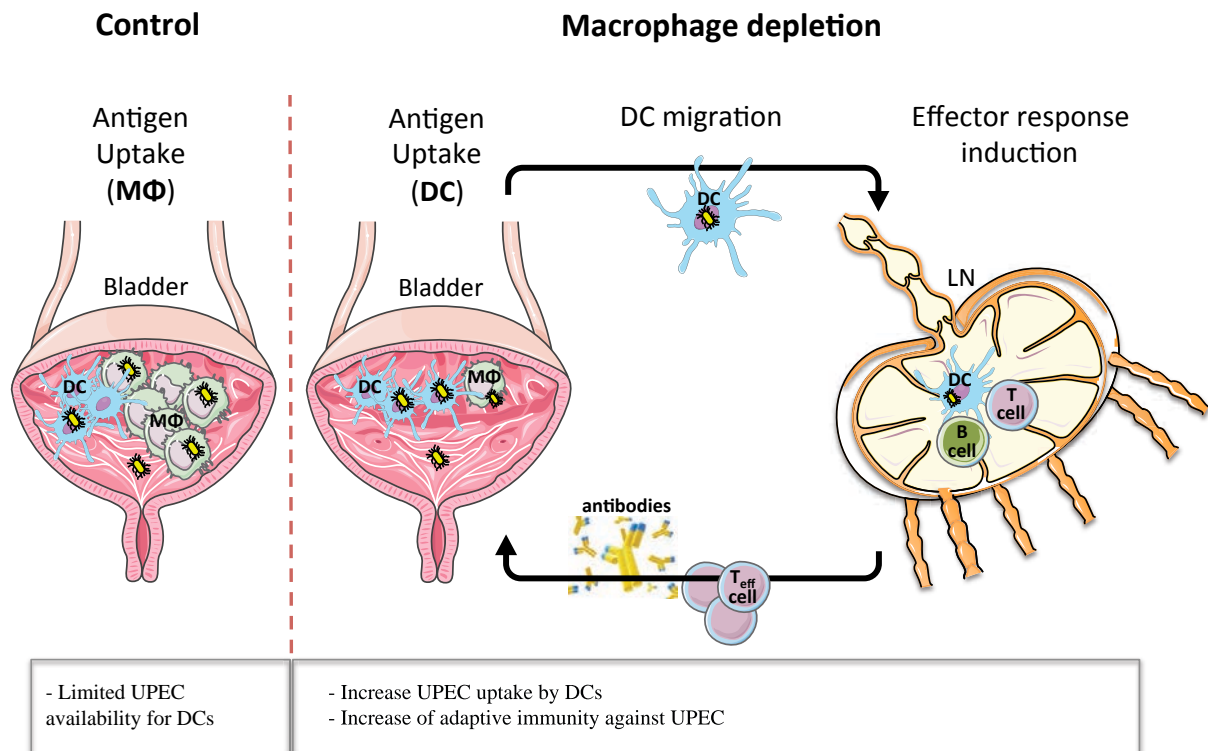


Figure 27: Proposed model for the role of macrophages during UTI. This scheme depicts our model regarding how an adaptive immune response is generated during UPEC infection in the presence (left, control) and absence (right, macrophage depletion) of macrophages. While in the control situation, macrophages acquire the majority of UPEC, limiting antigen acquisition by DCs and the generation of an adaptive immune response, macrophage depletion leads to an increase of UPEC-containing DCs and improves the adaptive immunity against UPEC.

Macrophages and their relationship with the adaptive immune response, are they always the “bad guys”?

In our study, we observed that macrophages negatively impact adaptive immunity generated during UTI (Mora-Bau et al., 2015), however, there are other examples describing the negative and positive impact of macrophages on adaptive immune responses. Thus, it may not be straightforward to describe the macrophage as a “good guy” or a “bad guy” during infection, and likely many variables impact the behavior and role of activated macrophages.

Good guys

Macrophages were thought to have little or no direct T cell priming capability; however, a recent report has shown that macrophages can present adenovirus particle-derived antigen to T cells *in vivo* in the presence or absence of DCs (Bernhard et al., 2015). Moreover, in the presence of both DCs and macrophages, this study shows that MHCI⁺ CD169⁺ macrophages were able to prime a more comprehensive immune reaction than cross-presenting DCs. Macrophages activated T cells specific for all the epitopes presented, whereas cross-presentation by DCs stimulated only those T cells that recognize immunodominant epitopes. Moreover, lymph node resident CD169⁺ macrophages have a prominent role in the priming of CTL-mediated antitumor immunity (Asano et al., 2011). CD169⁺ macrophages phagocytize dead tumor cells transported via lymphatic flow and subsequently cross-present tumor antigens to CD8⁺ T cells. In the absence of these macrophages, antitumor immunity is impaired (Asano et al., 2011). These results thus reveal a previously unknown role for CD169⁺ macrophages in the induction of adaptive immune responses, in the context of viral infection and tumor immunity.

Macrophages can also collaborate with DCs to promote health (Mazzini et al., 2014). Gut resident CD103⁺ DCs are responsible for inducing oral tolerance to fed antigens, however only the CX₃CR1⁺ macrophages and intestinal epithelial cells can efficiently capture these antigens from the lumen. How DCs acquire the antigens from the lumen then? It was recently demonstrated that CX₃CR1⁺ macrophages, quickly transfer these antigens after their capture to CD103⁺ DCs in a gap junction dependent manner (Mazzini et al., 2014). As we have observed that naïve bladders contain many CX₃CR1⁺ positive cells, it would be interesting to study whether resident macrophages and DCs can communicate with each other through these channels during UTI. For example, macrophages could transfer inhibitory signals to the DCs, as regulatory T cells transfer cAMP to effector CD4⁺ T cells to suppress their effector functions. Alternatively, if macrophages and DCs do not communicate during UTI, it might be that inducing their communication would lead to antigen transfer and an improved adaptive immune response.

Bad guys

In addition to sequestration of antigens from DCs (McLean et al. 1996; Kradin et al. 1999), macrophages can act as “bad guys” while interacting with the adaptive immune

response. This can be exemplified in the context of obesity-induced inflammation (Cho et al., 2014; Morris et al., 2013). Obesity-induced inflammation is associated with metabolic syndrome, cardiovascular disease, and the development of type 2 diabetes (Apovian et al., 2008; Lumeng and Saltiel, 2011; Ouchi et al., 2011). Adipose tissue macrophages possess all the functional activities of APCs and can promote antigen-specific T cell activation and T_H1 polarization of $CD4^+$ T cells in the fat of obese mice, helping to coordinate an adaptive immune response to obesity (Morris et al., 2013). Additionally, adipose tissue macrophages are required for the induction of effector/memory adipose tissue-specific $CD4^+$ T cells, which are involved in obesity-induced inflammation and insulin resistance (Cho et al., 2014). Deficient expression of MHC II in adipose tissue macrophages (which can no longer present antigens to $CD4^+$ T cells) leads to an improvement in glucose intolerance by increasing adipose tissue insulin sensitivity (Cho et al., 2014). Macrophages can also negatively impact the adaptive immune response to ovarian carcinoma, as it has been observed that with tumor cells, they attract regulatory T cells through the production of the chemokine CCL22 (Curiel et al., 2004).

During UTI, we demonstrated that macrophages act as the “bad guys” because they negatively impact the generation of an adaptive immune response against UPEC. Macrophages are professional phagocytic cells, however, their main role is to maintain the homeostasis of tissues, in part by secretion of cytokines to alert other immune cells to infection or inflammation. In the bladder, macrophages are the most abundant immune cells. They may phagocytize the majority of UPEC simply because they outnumber other phagocytes in the bladder at early timepoints. Thus, by fulfilling their role as phagocytic cells trying to clear pathogens, they exert a detrimental effect on the development of an adaptive immune response.

What is the nature of the immune response against UPEC?

We still do not completely understand how the adaptive immune system eliminates UPEC. Although we were not able to detect UPEC-specific antibodies above the limit of detection of our assay, others have identified that antibodies against bacteria are generated during infection (Hopkins et al., 1987; Svanborg-Eden and Svennerholm, 1978; Thumbikat et

al., 2006). Additionally, some studies evaluating vaccination strategies for UTI have measured the induction of a humoral response after vaccination and found that this response positively correlates with a positive outcome of the therapy (Asadi Karam et al., 2013; Langermann et al., 2000; Langermann et al., 1997). Thus, we hypothesized that the protection induced during UTI following a primary infection is mediated by an antibody response; however we also considered that T cells might play a direct role in the killing of infected cells. To directly evaluate the role of T cells during challenge, CD4⁺ and CD8⁺ T cells were depleted right before challenge infection and bacterial clearance was evaluated. We observed no difference in bacteria titers between T cell-depleted versus control treated mice, suggesting that T cells are not necessary to clear UPEC during challenge. Interestingly, when T cells were depleted before primary infection instead of challenge infection, mice were not protected against an UPEC challenge. The explanation for this may be that T cell depletion at the beginning of infection also impairs the generation of specific B cells and a humoral response, while T cell depletion right before challenge infection does not affect the humoral response already generated. Together, these results suggest that a humoral response is sufficient to confer protection during UPEC challenge.

Additionally, as UPEC is able to invade urothelial cells and even remain there for months as bacterial reservoirs, we hypothesized that a CD8⁺ T cell response is required to clear intracellular UPEC. We evaluated the role of CD8⁺ T cells indirectly by using Batf3 KO mice, which lack CD8 α ⁺ and CD103⁺ DCs impairing CD8⁺ T cell responses (Hildner et al., 2008). We did not observe any difference in bacterial clearance during challenge infection between WT and Batf3 KO mice, suggesting that CD8⁺ T cells are dispensable for fighting UPEC during a challenge infection. Importantly, it has been shown that CD8 α ⁺ and CD103⁺ DCs can develop in Batf3 mice, thus a CD8⁺ T cell response could be induced in these mice. To formally rule out a role for CD8⁺ T cells, we would need to measure whether they infiltrate during infection in the Batf3 mouse as well as to eliminate them directly prior to challenge infection.

Interestingly, we did not observe any difference in the reservoir either in these experiments, suggesting that once reservoirs are formed (which occurs early during infection (Mulvey et al., 2001)) they remained untouched by the immune system. To explain why the reservoirs are not affected by the adaptive immune response generated during UTI we propose two hypothesis: (1) UPEC-specific CD8⁺ T cells are not generated during UTI or (2)

UPEC-specific CD8⁺ T cells are generated, however, they do not recognize UPEC-infected cells as these cells are not presenting UPEC antigens. It would be interesting to perform *ex vivo* experiments and evaluate if CD8⁺ T cells obtained from UPEC-infected mice are able to kill UPEC-infected uroepithelial cells, as well as to evaluate the induction of UPEC-specific CD8⁺ T cells in infected mice through tetramer staining. Thus, while this is still a work in progress, we demonstrated that mice unable to induce a CD8⁺ T cell response (Batf3 KO experiments) and mice lacking T cells during UPEC challenge infection are able to clear infection as well as control mice, suggesting that even though we were unable to detect UPEC-specific antibodies during the course of our experiments, a humoral response during UTI likely mediates protection during (challenge) infection.

Can effective adaptive immunity be evoked from the bladder?

The early defense against bacterial infections, as well as the induction of an adaptive immune response, is dependent mainly on resident tissue cells (Iwasaki and Medzhitov, 2004; Medzhitov and Janeway, 2000), which initiate the immune response against invading pathogens. In this study, we showed that the bladder is equipped with different resident immune cell population including DCs, which are essential to mount an adaptive immune response. In addition to finding the right players to induce adaptive immune responses in the bladder, we observed that adaptive immunity can be primed from the bladder mucosa during UPEC infection (Mora-Bau et al., 2015). While we formally demonstrated the necessity for DCs, T and B cells in response to UTI, the fact that adaptive immune responses can be raised from the bladder is not a completely novel concept. Indeed, successful immunotherapy for bladder cancer is reliant upon robust adaptive immune responses. Bladder cancer is the fifth more common cancer in the US and Europe and with extraordinarily high health care costs (Ferlay et al., 2007; Siegel et al., 2013). Bacillus Calmette-Guerin (BCG) instillation is the primary treatment option for nonmuscle invasive bladder cancer patients after tumor resection and has been used for almost 40 years with high success rates (Dovedi and Davies, 2009; Fuge et al., 2015). Both UTI and bladder cancer have in common the fact that bacteria are introduced into the bladder lumen, as part of the infection process in UTI or as a therapy during bladder cancer. Interestingly, UPEC and BCG instillation appear to evoke very different immunological outcomes. Mouse experiments have demonstrated that following

BCG instillation into the bladder, BCG-specific CD8⁺ T cells are primed (Biot et al., 2012). Additionally, analysis of patient bladder biopsies has shown T cell infiltration during BCG therapy and that the degree of infiltration correlates with treatment response (Prescott et al., 1992). Moreover, the depletion of either CD4⁺ or CD8⁺ T cells abolishes BCG-mediated antitumor activity, suggesting that T cells play an important role during BCG immunotherapy (Ratliff et al., 1993). During UTI, we observe that an adaptive immune response is primed and confers protection against a challenge infection. However, this response neither prevents reinfection nor eliminates the bacterial reservoir established during primary infection. It has been shown that, during BCG treatment, live BCG can be found in the LNs and this phenomenon correlates with the priming of BCG-specific CD8⁺ T cells (Biot et al., 2012). Our preliminary experiments failed to detect the presence of live UPEC in the LNs during UTI. With this in mind, we could speculate that bladder APCs are unable to mount an efficient adaptive immune response and to achieve this response it would be necessary to have bacterial presence in the LNs. The necessity of the pathogen in the LNs to induce an adaptive immune response has also been shown during lung infection with *Mycobacterium tuberculosis*, suggesting that this phenomenon is not specific to the bladder and it is related to the nature of the pathogen (Chackerian et al., 2002; Wolf et al., 2008). The response generated during BCG treatment seems to be an “efficient” response as the presence of total T cells (T cell infiltrates in bladder biopsies) and/or BCG-specific T cells is associated with a better outcome for the disease (Biot et al., 2012; Prescott et al., 1992; Ratliff et al., 1993).

Why does the adaptive immunity generated during UTI “fail”?

Mice can develop an adaptive immune response during UTI however this response does not prevent reinfection. Therefore, the important questions are: why is the adaptive immune response generated during UTI not efficient? Why does this response not prevent recurrence? There are several possibilities to answer to these remaining open questions. The steps to induce an adaptive immune response during UTI include (1) UPEC acquisition in the bladder, (2) DC migration to the LNs, (3) priming of naive T and B cells, and (4) migration of effector cells to the bladder (Figure 28). We imagine that a failure in any of these steps could impair adaptive immunity generated during UTI. For example, during UPEC acquisition in the bladder, we demonstrated that macrophages sequester UPEC and limit bacteria acquisition

by DCs (Mora-Bau et al., 2015). In the next step, DC migration to the LNs is essential to induce an adaptive immune response, therefore a defect in DC migration could also add up to poor induction of adaptive immunity against UPEC. During tuberculosis, the generation of an adaptive immune response against *M. tuberculosis* is delayed, allowing bacterial growth and dissemination (Roberts and Robinson, 2014; Urdahl et al., 2011). Evidence suggests that this impaired response to *M. tuberculosis* is due to the fact that DC migration from the lung to the LNs is inhibited, which limits the initiation of an adaptive immune response (Roberts and Robinson, 2014; Urdahl et al., 2011). Following migration, DCs prime naive lymphocytes in the LNs to induce an adaptive immune response specific to the infecting pathogen. If the priming of UPEC-specific T cells is not efficient, the adaptive immune response will be impaired. As an example, during BCG immunotherapy, it has been observed that the induction of T_H1 T cells is associated with the success of therapy; however, a T_H2 response is associated with BCG failure (Nadler et al., 2003; Riemensberger et al., 2002). Finally, it is also possible that UPEC-specific effector cells do not migrate and/or infiltrate the bladder to fight infection. In addition, it is possible that, although specific T and B cells are generated, they are not long lasting and when women experience recurrence they do not have specific lymphocytes to fight the infection. Thus, there are many reasons that could give explain why the adaptive immune response induced during UTI is not efficient, leading to high rates of recurrence. Each of these possibilities needs to be tested in a rigorous experimental fashion, which is challenging given the number of different ways the immune response can fail. It is fundamental to continue studying how adaptive immunity is achieved against UPEC to understand what fails and how this failure can be overcome to improve the response.

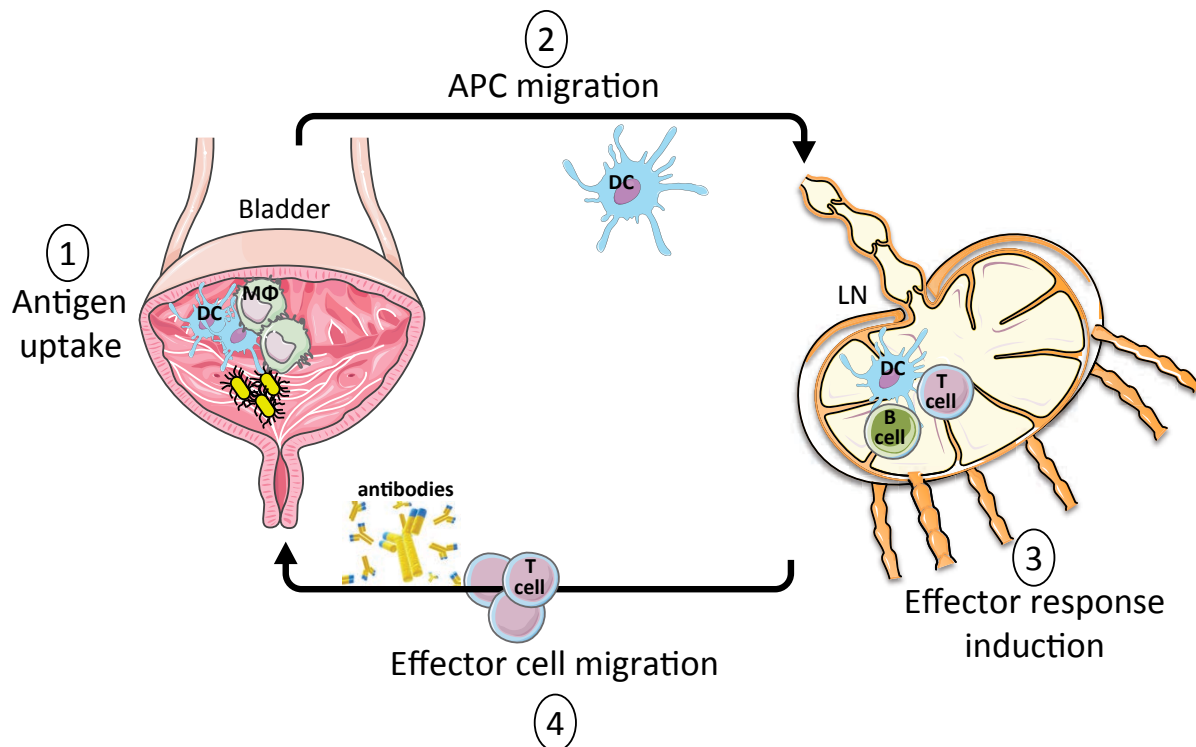


Figure 28: Proposed model for the generation of adaptive immunity during UTI. In this scheme, we illustrate the different required steps to induce adaptive immunity during UTI including: (1) antigen acquisition in the bladder mucosa, (2) DC migration to draining LNs, (3) priming of naïve lymphocytes and (4) migration of effector cells to the bladder.

In addition to these potential issues during the primary immune response, we have an additional layer of complexity: bacterial reservoirs. Different hypotheses have been proposed to explain UTI recurrence. These hypotheses include that UPEC strains colonize the gut and periodically migrate to the urinary tract (Chen et al., 2013), that the immune response to infection is suppressed by mast cell-derived IL-10 in the bladder (Chan et al., 2013), or that UPEC forms protected reservoirs in the bladder, reemerging at later time points after initial infection (Anderson et al., 2004; Mysorekar and Hultgren, 2006). It could also be a mix of the proposed explanations as they are not mutually exclusive. With this in mind, it would be fundamental to eliminate bacterial reservoirs from the bladder as we could be eliminating a major source of recurrence. To eliminate the reservoirs, a CD8⁺ T cell response would be necessary, however, the generation of UPEC-specific CD8⁺ T cells during UTI has not been demonstrated yet. Our challenge experiments in Batf3 KO mice suggest that CD8⁺ T cells are dispensable during UTI; however, further experiments are needed to fully demonstrate a role of these cells during infection. For example, through tetramer staining, we could evaluate if

UPEC-specific CD8⁺ T cells are generated during UTI. If these cells are generated, we can evaluate their ability to infiltrate the bladder. In addition, we could directly deplete CD8⁺ T cells and evaluate bacterial clearance during challenge and in UPEC reservoirs, as this is a more direct way to evaluate their role during infection than using Batf3 KO animals. Unfortunately, it may be that UPEC-specific CD8⁺ T cells are not generated during UTI, rendering the immune system incapable of detecting and eliminating infected cells. It is critical to remember, though, that although the rates of recurrence are high, more than 50% of UTI patients will not recur. We should consider studying these women, as it would be interesting to detect differences between them and women that tend to have recurrent infections to identify possible explanations for the failure of the response in those who recur. Along this line, there are some known genetic components in humans that may be associated with a major risk of UTI (Zaffanello et al., 2010). For example, it was observed that UTI-prone children express lower levels of CXCR1 (IL-8 receptor) than healthy children (Lundstedt et al., 2007). Additionally, low expression of TLR4 on neutrophils has been associated with asymptomatic bacteriuria in children suffering UTI with difficulties in clearing infection (Ragnarsdottir et al., 2007).

UTI affects millions of people every year and induce high health care costs (Foxman, 2010; Stamm and Norrby, 2001). Patients suffering from this infection are treated with antibiotics, however antibiotics do not prevent recurrence, which is a major problem among infected women. Importantly, antibiotic resistance is increasing, as well as the necessity of new therapeutic options (Flores-Mireles et al., 2015; Foxman, 2010; Hooton et al., 2004). Mechanism to increase the number of DCs and/or to increase the trafficking of T cells to the bladder could be considered as a way to improve the response to UTI. Additionally, we could consider that BCG instillation might be beneficial for the host response against UPEC as it is against tumor cells during BCG treatment for bladder cancer. As a short-term goal, to continue studying how an adaptive immune response is initiated and how it operates during UTI is critical. It is important to define, for example, which effector cells are fundamental for bacterial clearance during challenge and how we can eradicate UPEC reservoir. Once we have more knowledge about these fundamental questions it will be possible to define new therapeutic options to treat UTI patients and, very importantly, to prevent recurrence.

Materials and methods

Bacterial strains

The human UPEC cystitis isolate, UTI89 (kind gift from Scott Hultgren) (Mulvey et al., 2001), the fluorescent protein-expressing strains UTI89-RFP and UTI89-GFP (generated at Institut Pasteur (Mora-Bau et al., 2015)) were used for infection. Briefly, fluorescent bacteria were engineered using lambda red recombination (Chaveron et al., 2000) to introduce an *aphA-marsRFP* or *bla-GFP* cassette in the UTI89 chromosome at the *attB* lambda phage integration site. UTI89 is sensitive to antibiotics, UTI89-RFP is resistant to kanamycin, and UTI89-GFP is resistant to ampicillin, Top52 is sensitive to antibiotics. Bacteria were grown overnight in static cultures at 37°C in Luria-Bertani broth (LB) in the presence of antibiotics (kanamycin 50 mg/mL or ampicillin 100 mg/mL) when appropriate.

Cell lines and *in vitro* invasion assay

The mouse uroepithelial cell line NUC-1 (De Boer et al., 1993) was used to evaluate *in vitro* invasion of the newly generated UTI89 fluorescent strains (UTI89-GFP and UTI89-RFP). Fifty thousand NUC-1 cells were infected with UTI89, UTI89-GFP, or UTI89-RFP at increasing MOIs of bacteria. Thirty minutes post-infection, cells were washed, lysed, and serial dilutions were plated in LB plates or antibiotic plates as appropriate. Percentage of invasion was calculated by dividing the number of bacteria inside the cells by the inoculum x 100.

Mice and infections

Female C57BL/6 mice between 6 and 8 weeks old were from The Jackson Laboratory or Charles River. CD11c-DTR mice were a kind gift from Marc Lecuit and Claude LeClerc (Institut Pasteur). RAG2^{-/-} mice and CD3^{-/-} mice were a kind gift from Antonio Freitas (Institut Pasteur). Batf3^{-/-} mice were purchased from The Jackson Laboratory. Briefly, mice anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine were infected with 10⁷ colony-forming units (CFU) of TOP52 or UTI89 strains (UTI89, UTI89-GFP or UTI89-RFP) in 50 µL PBS via a catheter introduced into the urethra (Hung et al., 2009). In the inoculum escalating experiment, mice received 10⁷, 10⁸ or 10⁹ CFU in 50 µL PBS. To calculate CFU,

bladders were aseptically removed and homogenized in 1 mL PBS. Serial dilutions were plated on LB agar, with or without antibiotics, as required and CFU in each plate were counted 16-18 hours later. For challenge infection experiments, mice were infected with one of the two fluorescent strains of UTI89, expressing antibiotic resistance (kanamycin or ampicillin). Once the primary infection cleared, 3 to 4 weeks later, mice were infected with 10^7 CFU of an isogenic UTI89 strain with a different antibiotic resistance (See Figure 10). The strain used for the challenge infection was determined by that used in the primary infection, such that the antibiotic resistances were different between the primary and challenge infection, *e.g.*, UTI89-GFP for the primary and UTI89-RFP for the challenge infection. Importantly, both strains were used as the primary or the challenge strain in different experiments. Resolution of primary infection was monitored by plating urine every 5-6 days on antibiotic-containing plates.

Irradiation, bone marrow cell transfer, diphtheria toxin treatment

C57Bl/6 mice were irradiated with a single dose of 5-6 gray in an x-ray irradiator at 6 weeks of age. Animals were reconstituted with $1.6\text{--}3.2 \times 10^6$ total bone marrow cells from CD11c-DTR mice 6 hours after irradiation. Mice were allowed to reconstitute for a minimum of 12 weeks and reconstitution was evaluated by flow cytometry of congenic markers. 48 and 24 hours prior to infection, mice were administered 4 ng/g of diphtheria toxin I.V. Depletion efficiency was tested in each batch of chimeric mice prior to experimentation.

Flow cytometry of bladder tissue

At indicated timepoints, bladders were removed and minced with dissection scissors into tubes containing 1 mL of digestion buffer (0.34 U/mL of Liberase TM (Roche) and 100 mg/mL of DNase in PBS) kept at 4°C. Minced tissue was then incubated at 37°C. Tubes were vigorously shaken by hand every 15 minutes. 45 minutes to 1 hour post-incubation, when the tissue had a glassy, transparent appearance and was almost entirely digested, digestion was stopped by adding several mL of PBS supplemented with 2% FBS and 0.2 mM EDTA. The bladder digest was then passed through a 100 μm cell strainer to obtain a single cell suspension. Gentle pressure was applied to any tissue remaining in the strainer. Samples were washed, Fc receptors were blocked by adding 1:50 Fc block during 15 minutes, and stained

with antibodies listed in Table 2. Total cell counts in the bladder were determined by the addition of AccuCheck Counting beads (Invitrogen) to a known volume of sample after staining, just prior to cytometer acquisition. Samples were acquired on a BD LSRFortessa using DIVA software and data were analyzed by FlowJo (Treestar) software. Gating strategies for all cell populations except for neutrophils are depicted in figure 13. Neutrophils were identified as MHC II⁻, CD11b⁺, Ly6G⁺, Ly6C⁻, SiglecF⁺, and F4/80⁻.

Flow cytometry of blood

To identify cell populations in the circulation, whole blood was incubated with BD PharmLyse, (BD Bioscience) and subsequently stained with antibodies indicated in the Table 2. Samples were acquired on a BD LSRFortessa using DIVA software and data were analyzed by FlowJo (Treestar) software. Total cell counts in the blood were determined by the addition of AccuCheck Counting beads (Invitrogen) to 10 μ L of whole blood diluted in 1-step Fix/Lyse Solution (eBioscience).

Monocyte Bead labeling

In vivo bead labeling of classical and nonclassical monocytes was performed as previously described (Tacke et al., 2006). Briefly, classical monocytes were labeled by I.V. administration of 200 μ L clodronate liposomes to transiently deplete all monocytes and then by I.V. injection of 1 μ M nondegradable fluorescent particles 24 hours later. Nonclassical monocytes were labeled by injection of 1 μ M nondegradable fluorescent particles without prior monocyte depletion. Labeling efficiency was confirmed by flow cytometry.

Immune cell depletion

To deplete monocytes, wildtype C57BL/6 mice received I.V. injection of 200 μ L of clodronate liposomes (or PBS control liposomes) 15-18 hours prior to infection (Van Rooijen and Sanders, 1994). To deplete bladder-resident macrophages anti-CSF1R antibody (2 mg/mL, clone AFS98, eBioscience) was used. Animals received two I.V. injections, on two consecutive days, of anti-CSF1R antibody or isotype control (clone eBR2a, eBioscience). We administered 400 μ g/mouse on day 1 and 200 μ g/mouse on day 2, to decrease the impact on

circulating monocytes. To deplete T cells, 100 µg of CD4 (clone GK1.5, Bio X Cell) and 100 µg of CD8 (clone YTS 169.4, Bio X Cell) per mouse were injected together intraperitoneally 24 hours prior to primary infection. As a control, 200 mg of isotype control (clone LTF-2, Bio X Cell) per mouse was injected intraperitoneally 24 hours prior to primary infection. The depletion was repeated 5 days post-infection, and once a week to maintain the depletion until the moment of challenge infection.

Luminex MAP analysis

Mice were infected with UTI89 and bladders removed 24 hours P.I. Bladders were homogenized with a handheld tissue grinder in 1 mL PBS on ice. After removal of a 100 µL aliquot to calculate CFU by serial dilution, bladder homogenates were clarified by microcentrifugation (13K, 4, 5 minutes) and stored at -80°C until assessment by Luminex Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Premixed 32-Plex, according to the manufacturer's recommendations (Merck Millipore) (11 paper). All samples were assessed together to avoid inter-assay variability. Just prior to analysis, after thawing, samples were centrifuged a second time to remove any cell debris.

ELISA

Urine and plasma samples were collected during the course of experiments and preserved at -20°C. At the time of sacrifice, bladder lysates were collected and preserved at -20°C. Total UPEC-specific IgA was evaluated by a homemade ELISA, which was developed using the mouse IgA total ELISA Ready-SET-go (eBioscience). 96-well microwell plates (Nunc) were coated with UPEC lysate (50 ug/mL in coating buffer) and incubated overnight at 4°C. Then, plates were washed and blocked with blocking buffer. After blocking, plates were washed, incubated with urine and later incubated with HRP-conjugated anti-mouse IgA detection antibody followed by incubation with the substrate solution. Plates were read in LabSystems Multiskan MS plate reader.

Statistical analysis

GraphPad Prism was used to evaluate statistical significant. Graphs depict medians and statistical significance was determined by the nonparametric Mann-Whitney test.

Table 3: Antibodies used for flow cytometry.

Molecule	Clone	Vendor
CD3	145-2C11	BD Bioscience
CD4	RM4-5	BD Bioscience
CD4	GK1.5	BD Bioscience
CD8	53-6.7	BD Bioscience
CD45	30-F11	BD Bioscience
CD64	X54-5/7.1	BD Bioscience
CD103	M290	BD Bioscience
CD115	AFS98	eBioscience
CD11b	M1/70	BD Bioscience
CD11c	N418	eBioscience
c-Kit	2B8	BD Bioscience
F4/80	CI:A3-1	AbD Serotec
$\gamma\delta$ TCR	GL3	eBioscience
Gr1	RB6-8C5	BD Bioscience
IgE	R35-72	BD Bioscience
Ly6G	1A8	BD Bioscience
Ly6C	AL-21	BD Bioscience
MHC-II (I-A/I-E)	M5/114.15.2	eBioscience
NK 1.1	PK136	BD Bioscience
Nkp46	29A1.4	BD Bioscience
SiglecF	E50-2440	BD Bioscience

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RESEARCH ARTICLE

Macrophages Subvert Adaptive Immunity to Urinary Tract Infection

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Abstract

Urinary tract infection (UTI) is one of the most common bacterial infections with frequent recurrence being a major medical challenge. Development of effective therapies has been impeded by the lack of knowledge of events leading to adaptive immunity. Here, we establish conclusive evidence that an adaptive immune response is generated during UTI, yet this response does not establish sterilizing immunity. To investigate the underlying deficiency, we delineated the naïve bladder immune cell compartment, identifying resident macrophages as the most populous immune cell. To evaluate their impact on the establishment of adaptive immune responses following infection, we measured bacterial clearance in mice depleted of either circulating monocytes, which give rise to macrophages, or bladder resident macrophages. Surprisingly, mice depleted of resident macrophages, prior to primary infection, exhibited a nearly 2-log reduction in bacterial burden following secondary challenge compared to untreated animals. This increased bacterial clearance, in the context of a challenge infection, was dependent on lymphocytes. Macrophages were the predominant antigen presenting cell to acquire bacteria post-infection and in their absence, bacterial uptake by dendritic cells was increased almost 2-fold. These data suggest that bacterial uptake by tissue macrophages impedes development of adaptive immune responses during UTI, revealing a novel target for enhancing host responses to bacterial infection of the bladder.

Author Summary

Urinary tract infection is a common infection with a high propensity for recurrence. The majority of infections are caused by uropathogenic *E. coli*, a growing public health concern with increasing prevalence of antibiotic resistant strains. Finding therapeutic options that circumvent the need for antibiotics, while boosting patients' immune response to infection is desirable to counteract further increases in antibiotic resistance and to provide long-lasting resistance to infection. Currently, little is known about how adaptive immune

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responses, which typically prevent recurrent infection in other organs, arise from the bladder during urinary tract infection. Here, we investigated the initial interactions between immune cell populations of the bladder and uropathogenic *E. coli*, finding that macrophages are the principal cell population to engulf bacteria. Interestingly, these same cells appear to inhibit the development of adaptive immunity to the bacteria, as their depletion, prior to primary infection, results in a stronger immune response during bacterial challenge. We found that in the absence of macrophages, dendritic cells, which are the most potent initiators of adaptive immunity, are able to take up more bacteria for presentation. Our study has revealed a mechanism in which specific immune cells may act in a manner detrimental to host immunity.

Introduction

Urinary tract infection (UTI) is one of the most common bacterial infections, impacting more than 130 million people annually worldwide [1,2]. The principal causative agent is uropathogenic *Escherichia coli* (UPEC), accounting for more than 75% of all community acquired infections, particularly among a seemingly healthy population (e.g., premenopausal women) [1]. In uncomplicated UTI (i.e., cystitis), nearly half of all women infected will experience recurrence [3]. Currently, there is little consensus in the field regarding the underlying causes of the high rate of recurrence. Mechanisms previously proposed to explain this phenomenon include that UPEC forms protected reservoirs in the bladder, reemerging at later time points after initial infection [4,5]; that UPEC strains colonize the gut and periodically migrate to the urinary tract [6]; or that the immune response to infection is suppressed by mast cell-derived IL-10 in the bladder [7].

The innate immune response to UPEC infection is characterized by robust cytokine and chemokine expression, leading to rapid neutrophil and monocyte infiltration and subsequent bacterial clearance [8–12]. Depletion of both neutrophils and monocytes, by Gr1 antibody treatment [13], leads to increased bacterial burden, whereas a reduction in circulating neutrophils alone decreases bacterial burden, suggesting that monocytes help eliminate bacteria in the bladder [10,11]. A recent study demonstrated that innate immune cell crosstalk is necessary for a coordinated innate response, whereby resident macrophages, responding to signals from infiltrating monocytes, induce MMP9 expression in neutrophils, in turn facilitating their trans-urothelial migration [14]. This mechanism likely works in concert with cytokine and chemokine expression from infected urothelium that mediates neutrophil recruitment and trans-urothelial migration [9].

The mechanisms involved in the initiation of adaptive immunity, and indeed the full nature of the response generated from the bladder during UTI, remain unclear [15]. The majority of studies have focused on innate immunity to UTI, such as neutrophil or monocyte infiltration, while only a limited number of studies have focused on adaptive immune mechanisms [16]. For example, UPEC-specific antibodies arise during UTI in mice, non-human primates, and human patients, and can inhibit UPEC binding to urothelial cells *in vitro* [17–19]. With respect to the role of effector cells, only one study has examined the induction of antigen-specific antibody and T cell responses after UPEC infection, demonstrating that transfer of serum or T cells from infected animals limits infection in naïve mice [19].

In this study, we investigated the initiation of adaptive immunity to UPEC to determine whether defects exist preventing the induction of sterilizing immunity. We conclusively demonstrated that adaptive immune responses are generated in response to UPEC infection;

however, they are insufficient to prevent reinfection. We performed the first systematic analysis of the tissue-resident immune cell compartment in the steady state bladder of mice and investigated the role of macrophages, and their precursors, in the adaptive immune response during UTI. Strikingly, macrophage depletion, prior to primary infection, improved adaptive immune responses to challenge infection in a macrophage-replete environment. We observed that upon infection, macrophages were the principal population, among the antigen presenting cells, to acquire UPEC early in infection, and in their absence, bacterial uptake by dendritic cells (DCs) was increased. These data support a model in which bladder-resident macrophages sequester bacteria, consequently limiting adaptive immune responses, and provides an explanation for the failure of the immune system to respond effectively to UPEC infection.

Results

UPEC infection primes an adaptive immune response mediated by DCs

Surprisingly, no study has directly tested the necessity of an adaptive immune response to limit UPEC reinfection or the role of specific components of the adaptive immune system in generating these responses. We employed a model of UPEC-induced cystitis in which 10^7 colony-forming units (CFU) of UPEC isolate UTI89, made resistant to either ampicillin or kanamycin, were instilled intravesically into 7–8 week old female wildtype C57Bl/6 or C57Bl/6 RAG2^{-/-} mice [20]. Animals were sacrificed at 24 hours post-infection (P.I.) to assess bacterial burden or monitored for bacteriuria to evaluate the resolution of acute infection, defined by the absence of bacteria in the urine. Three to four weeks later, when the mice had resolved the primary infection, animals were challenged with 10^7 CFU of an isogenic UPEC strain, resistant to the antibiotic not employed for primary infection, and sacrificed 24 hours P.I. to evaluate bacterial clearance (Fig 1A). Importantly, the use of isogenic UPEC strains, differing only by antibiotic resistance and fluorescent marker, permitted differentiation between quiescent bacteria residing in reservoirs established during primary UPEC infection [5] and the challenge strain. Of note, this distinction has not been made in previous reports, and thus it has remained unclear whether bacteria measured in the bladder after challenge infection derive from the primary or challenge infection, or represent a mixture of both infections [19]. After UPEC challenge in wildtype mice, we observed a >2 log reduction in CFU of the challenge UPEC strain compared to the bacterial burden 24 hours after primary infection (Fig 1B). By contrast, the bacterial burden after challenge infection in RAG2^{-/-} mice was similar to that after primary infection (Fig 1B).

As DCs are the principal cells to present antigen to lymphocytes, we investigated their role in inducing an adaptive immune response following a primary infection. Utilizing CD11c-DTR chimeric mice, we depleted CD11c-expressing DCs, prior to primary infection, by administration of two doses of diphtheria toxin (S1 Fig). As previously reported, diphtheria toxin treatment also impacted the number of tissue resident macrophages (S1 Fig and [21]); however, this reduction was minimal and the number of macrophages present in toxin-treated mice was within the range of normal variance (Table 1). Twenty-four hours after depletion, we infected mice as described in Fig 1A, and followed resolution of infection by assessing bacteriuria. Upon resolution of the primary infection, mice were challenged with an isogenic UTI89 strain, as described above. To measure the bacterial burden following primary infection in the chimeric mice, an additional cohort of naïve, untreated chimeric mice received a primary infection at the same time as the infected animals received the challenge infection (Fig 1C, 1° group). We assessed bacterial burden at 24 hours following primary or challenge infection and observed that animals treated with PBS were better able to clear UPEC after challenge compared to DC-depleted animals (Fig 1C). Together, these results suggest that the reduction in CFU observed

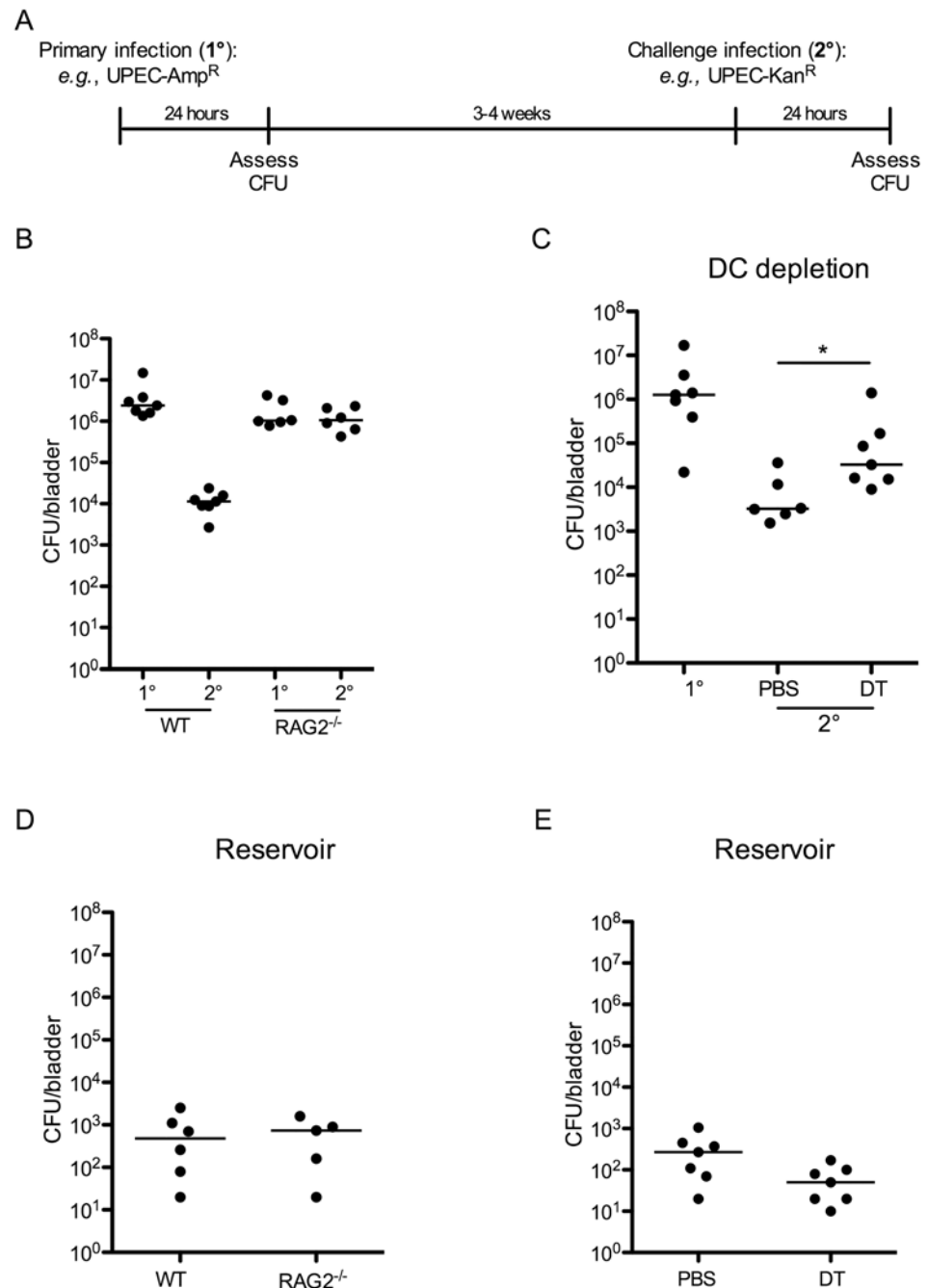


Fig 1. An adaptive immune response is necessary for bacterial clearance during UPEC challenge infection. (A) Experimental scheme used in the study. (B) Female C57Bl/6 (WT) or RAG2^{-/-} mice were instilled with UTI89 and sacrificed 24 hours P.I. (1°) or challenged with an isogenic UPEC strain carrying a different antibiotic marker and sacrificed 24 hours P.I. (2°) to evaluate bacterial burden. (C) 12 weeks post-reconstitution, chimeric CD11c-DTR mice were treated with PBS or diphtheria toxin (DT) to eliminate DCs and infected with UTI89 24 hours post treatment. Mice were challenged with the isogenic UPEC strain and sacrificed 24 hours P.I. to measure CFU/bladder (2°). At the time of the challenge infection in (C), an additional group of naïve CD11c-DTR chimeric mice was infected with UTI89 to evaluate CFU after a primary UPEC infection (1°). (D-E) Graphs depict CFU/bladder of the primary strain from the infections in (B) and (C), respectively. Each dot represents one mouse, lines are medians. Experiments were performed 2 times with 5–7 mice per group in each experiment. *p = 0.0221, Mann-Whitney.

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Table 1. Immune cell populations in naïve bladders.

Naïve bladder	
Cell subset	Cell number ^a
CD45 ⁺	35714 ± 11161
Macrophages	9035 ± 3480
CD11b ⁺ DCs	4046 ± 1785
CD103 ⁺ DCs	1011 ± 443
Monocytes	720 ± 467

^a Cell numbers are displayed as the mean ± the standard deviation. Values are derived from at least 8 mice, analyzed in separate experiments.

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after a challenge infection is mediated by an adaptive immune response, dependent upon DCs and lymphocytes. Interestingly, this response to UPEC reduces bacterial burden but does not prevent re-infection after challenge (Fig 1 and [19]).

Finally, in our model, approximately 10% of the total CFU measured after a challenge infection arose from the primary infecting strain, representing the reservoir formed during infection. These bacteria appeared to be protected from host clearance mechanisms, as no differences in the number of bacteria present in the reservoir between wildtype and RAG2^{-/-} mice (Fig 1D), or PBS and DT-treated CD11c-DTR mice were observed (Fig 1E).

The bladder contains a diverse immune cell repertoire

To understand how adaptive immune responses are initiated in the bladder, we began by investigating the resident immune cell compartment of the bladder. Earlier studies have described bladder-resident DCs [22–25], however, a comprehensive analysis of all immune cell populations has not been previously performed. Thus, we executed a systematic analysis of the bladder-resident CD45⁺ immune cell compartment. Bladders from naïve C57Bl/6 mice were enzymatically digested and immunostained (Materials and Methods, Table 2). As the bladder's broad autofluorescent signal interfered with immune cell detection, we developed a gating strategy to exclude nonhematopoietic autofluorescent cells during analysis (S2A–S2C Fig). Antigen presenting cells (APCs), defined as MHC II⁺, comprised the majority of CD45⁺ cells (69% ± 7.5 of CD45⁺ cells, Fig 2A and 2E). Macrophages, delineated by CD64 [26,27] and F4/80 co-expression, were by far the largest APC population (~40% of CD45⁺ cells) (Fig 2B and 2E). The CD11b⁺ and CD103⁺ dendritic cell (DC) subsets represented 15% and 5% of CD45⁺ cells, respectively (Fig 2B and 2E). Within the MHC II⁺ CD11b⁺ gate, we identified NK1.1⁺ NK cells, CD11b^{lo-int} cKit⁺ IgE⁺ mast cells [28], CD3⁺ CD4⁺, and CD3⁺ γδ⁺ T cells, but never observed CD8⁺ T cells in naïve bladders (Fig 2C and 2E). Recently, it was reported that classical Ly6C⁺ monocytes constitutively traffic into naïve nonlymphoid tissues, such as skin, in the steady state [29,30]. Accordingly, in the MHC II⁺ CD11b⁺ gate, we identified resident Ly6C⁺ monocytes as well as SiglecF⁺ eosinophils [31] (Fig 2D and 2E). Notably, no neutrophils were observed in naïve bladders.

Infiltrating classical monocytes differentiate to macrophages during infection

In addition to neutrophils, monocytes infiltrate the bladder upon UPEC infection [8,11]. To determine the fate of infiltrating monocytes, we employed *in vivo* labeling of circulating

Table 2. Antibodies used in this study.

Molecule	Clone	Vendor
CD3	145-2C11	BD Bioscience
CD4	RM4-5	BD Bioscience
CD4	GK1.5	BD Bioscience
CD8	53–6.7	BD Bioscience
CD45	30-F11	BD Bioscience
CD64	X54-5/7.1	BD Bioscience
CD103	M290	BD Bioscience
CD115	AFS98	eBioscience
CD11b	M1/70	BD Bioscience
CD11c	N418	eBioscience
c-Kit	2B8	BD Bioscience
F4/80	Cl:A3-1	AbD Serotec
gammadelta TCR	GL3	eBioscience
Gr1	RB6-8C5	BD Bioscience
IgE	R35-72	BD Bioscience
Ly6G	1A8	BD Bioscience
Ly6C	AL-21	BD Bioscience
MHC-II (I-A/I-E)	M5/114.15.2	eBioscience
NK 1.1	PK136	BD Bioscience
Nkp46	29A1.4	BD Bioscience
SiglecF	E50-2440	BD Bioscience

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monocytes to monitor their entry into infected bladders [8,32]. Mice were infected 24 hours after labeling circulating classical or nonclassical monocytes and sacrificed at 4, 24, and 48 hours P.I., for analysis by flow cytometry. In line with observations from other infection models [33,34], a greater number of classical monocytes infiltrated the bladder over time than non-classical monocytes (Fig 3A, note the scales of the y-axes). In the classical labeling protocol, a majority of infiltrated bead⁺ cells upregulated CD11c, MHC II, and CD64 expression, and downregulated CD11b and Ly6C from 24 to 48 hours P.I., phenotypically resembling resident macrophages (gray histograms) (Fig 3B). The percentage of bead⁺ cells that were identified as macrophages increased from 24 to 48 hours, while only 10% of all bead⁺ cells had a DC phenotype (Fig 3C), supporting the conclusion that infiltrating classical monocytes predominantly differentiate into macrophages during UTI.

Depletion of bladder-resident macrophages improves adaptive immunity to UPEC

During infection, infiltrating monocytes increased the already substantial macrophage compartment, thus we hypothesized that monocyte-derived and/or resident macrophages might play an important role during UPEC infection. To test this hypothesis, we depleted each population separately to determine the impact on bacterial burden. Circulating monocytes, but not bladder-resident macrophages, were depleted with clodronate liposomes [35] (S3A and S3B Fig), and mice were infected 15–18 hours later with UPEC. Mice were sacrificed 24 hours P.I. to determine CFU. Monocyte-depleted animals had a small (<1-log) but significant improvement in bacterial elimination 24 hours P.I. (Fig 4A). This difference, however, was lost if mice were infected 24 hours post-clodronate treatment, when monocytes have begun to repopulate

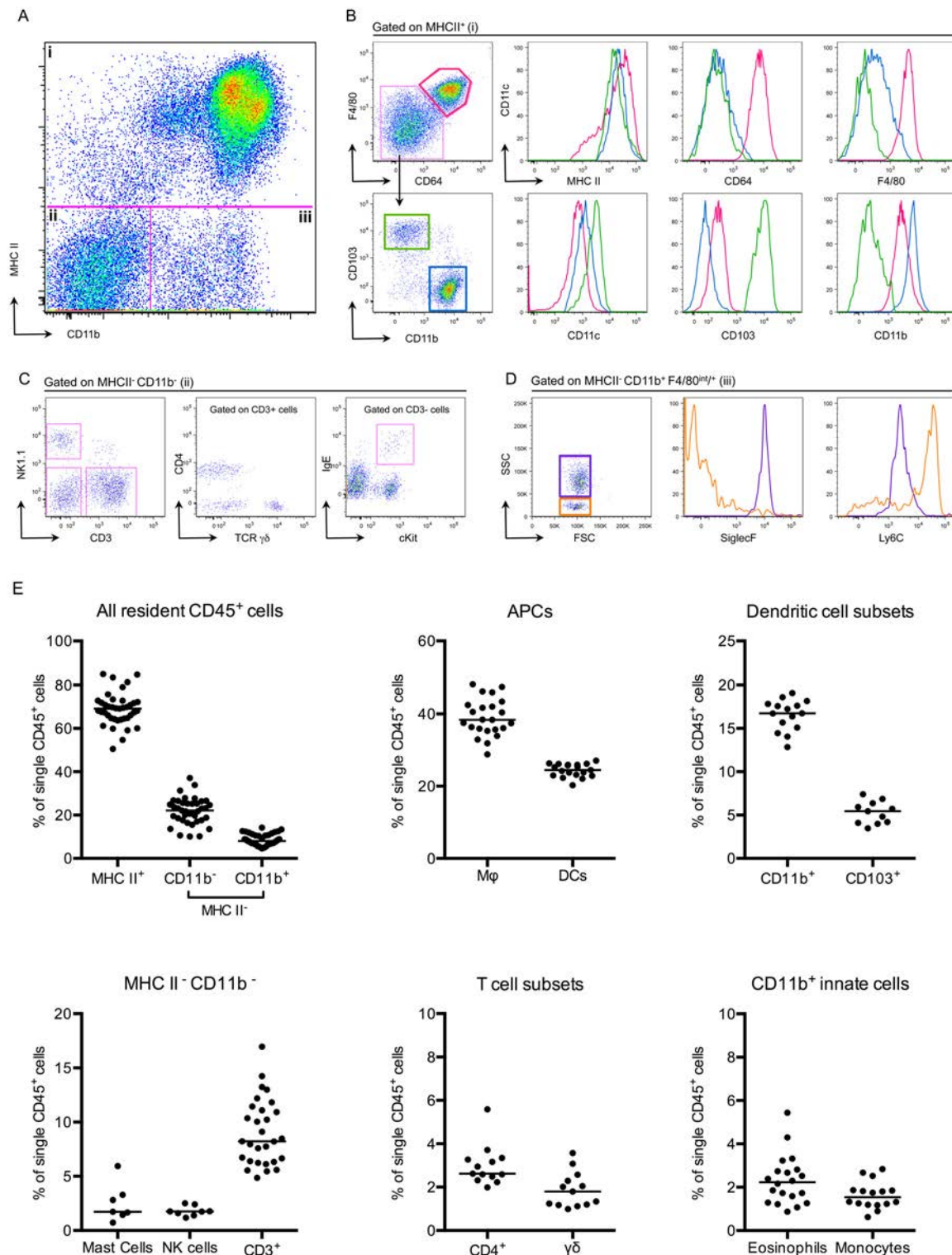


Fig 2. The bladder contains a diverse immune cell repertoire. Naïve bladders from female C57Bl/6 mice were digested for flow cytometry. Single cell preparations were stained with antibodies indicated in Table 2. (A) Single, CD45⁺ cells were gated into 3 groups (i-iii) according to their CD11b and MHC II expression levels. (B) MHC II⁺ cells from gate (i) were divided into 3 populations, F480⁺ CD64⁺ macrophages (pink gate), CD11b⁺ DCs (blue gate) and CD103⁺ DCs (green gate). The expression levels of MHC II, CD64, F4/80, CD11c, CD103, and CD11b are depicted in the histograms (macrophages—pink lines, CD11b⁺ DCs—blue lines, CD103⁺ DCs—green lines). (C) MHC II⁻ CD11b⁻ cells from gate (ii) were subdivided by their expression of CD3 and NK1.1. CD3⁺ cells were divided into CD4⁺ and TCR $\gamma\delta$ ⁺ and the CD3⁻ gate shows cKit⁺ IgE⁺ mast cells. (D) The dot plot, from gate (iii) depicts MHC II⁻ CD11b⁺

F480^{int/+} eosinophils (purple gate) and monocytes (orange gate). Histograms depict expression levels of SiglecF and Ly6C from gate (iii) (eosinophils—purple lines, monocytes—orange lines). (E) Graphs depict immune cell populations in the naïve bladder as percentage of all CD45⁺ cells. Representative cytometry dot plots and histograms from different individual bladders are depicted, with the exception of the mast cell plot in (C), which depicts three pooled bladders due to the low number of mast cells. In (E), each dot represents one bladder, lines are medians, plots are pooled from three or more experiments.

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the circulation, suggesting that monocyte depletion has a transient impact on bacterial burden. Supporting this conclusion, there were no differences in bacterial burden 24 hours P.I. in CCR2^{-/-} mice (S4 Fig), which have greatly reduced numbers of circulating monocytes [33], as compared to wildtype mice.

Bladder-resident macrophages were eliminated by administration of anti-CSF1R depleting antibody 24 hours prior to infection (S3C Fig) [36]. Importantly, the anti-CSF1R antibody also targeted monocytes, however these cells were not completely eliminated from circulation at the time of infection (compare S3A Fig to S3D Fig). Mice depleted of macrophages had a similar bacterial burden at 24 hours P.I. compared to non-depleted mice (Fig 4B) suggesting that the absence of macrophages does not impact UPEC clearance at early time points P.I.

As macrophage depletion had no impact on the primary infection, we considered whether their absence might influence the generation of an adaptive immune response. Indeed, macrophages can impact adaptive immunity via cytokine secretion or antigen sequestration. To directly test the influence of monocytes and macrophages on the generation of adaptive immunity during UTI, we depleted each of these cell types, as above. To address the role of monocytes, mice were depleted by clodronate treatment, infected, and subsequently challenged with an isogenic UTI89 strain and sacrificed at 24 hours P.I. to determine CFU, as in (Fig 1A). We did not observe a difference in bacterial burden at 24 hours post-challenge (Fig 4C) or in reservoir formation (S5 Fig), suggesting that the absence of monocytes, before primary infection, did not influence bacterial clearance after challenge infection. Further, these data demonstrate that the small improvement observed in bacterial clearance at 24 hours post-primary infection after clodronate treatment (Fig 4A), did not influence the development of an adaptive immune response to the bacteria.

To determine whether resident macrophages influence bacterial clearance after challenge, we treated mice with anti-CSF1R and infected with UPEC. Upon resolution of the primary infection, mice were challenged with an isogenic UTI89 strain and bacterial burden determined 24 hours post-challenge. In the course of the experiment, macrophage depletion did not impact reservoir formation (S5 Fig). Despite similar clearance during the primary infection, we observed a surprising reduction of nearly 2 orders of magnitude in CFU after challenge infection of mice depleted of macrophages prior to the primary infection as compared to control isotype-treated and untreated (infected and challenged, but not receiving antibody injection) mice (Fig 4D). Importantly, at the time of the challenge infection, macrophages had repopulated the bladder in depleted animals, ruling out the possibility that bacterial burden was influenced by the absence of macrophages during challenge infection (S3E Fig).

To test whether this improvement in bacterial clearance in the absence of macrophages was dependent upon components of the adaptive immune system, we depleted macrophages in RAG2^{-/-} mice. We observed no difference in the CFU per bladder after UPEC challenge between macrophage-depleted or control treated RAG2^{-/-} mice (Fig 4E). To specifically assess the necessity of T cells, we depleted macrophages in mice that had been treated with CD4 and CD8 depleting antibodies prior to primary infection. We observed fewer UPEC post-challenge only in macrophage-depleted mice that were replete of their T cells. However, mice depleted of CD4⁺ and CD8⁺ T cells did not demonstrate improved bacterial clearance after challenge independently of macrophage depletion (Fig 4F). Together, these results support the conclusion

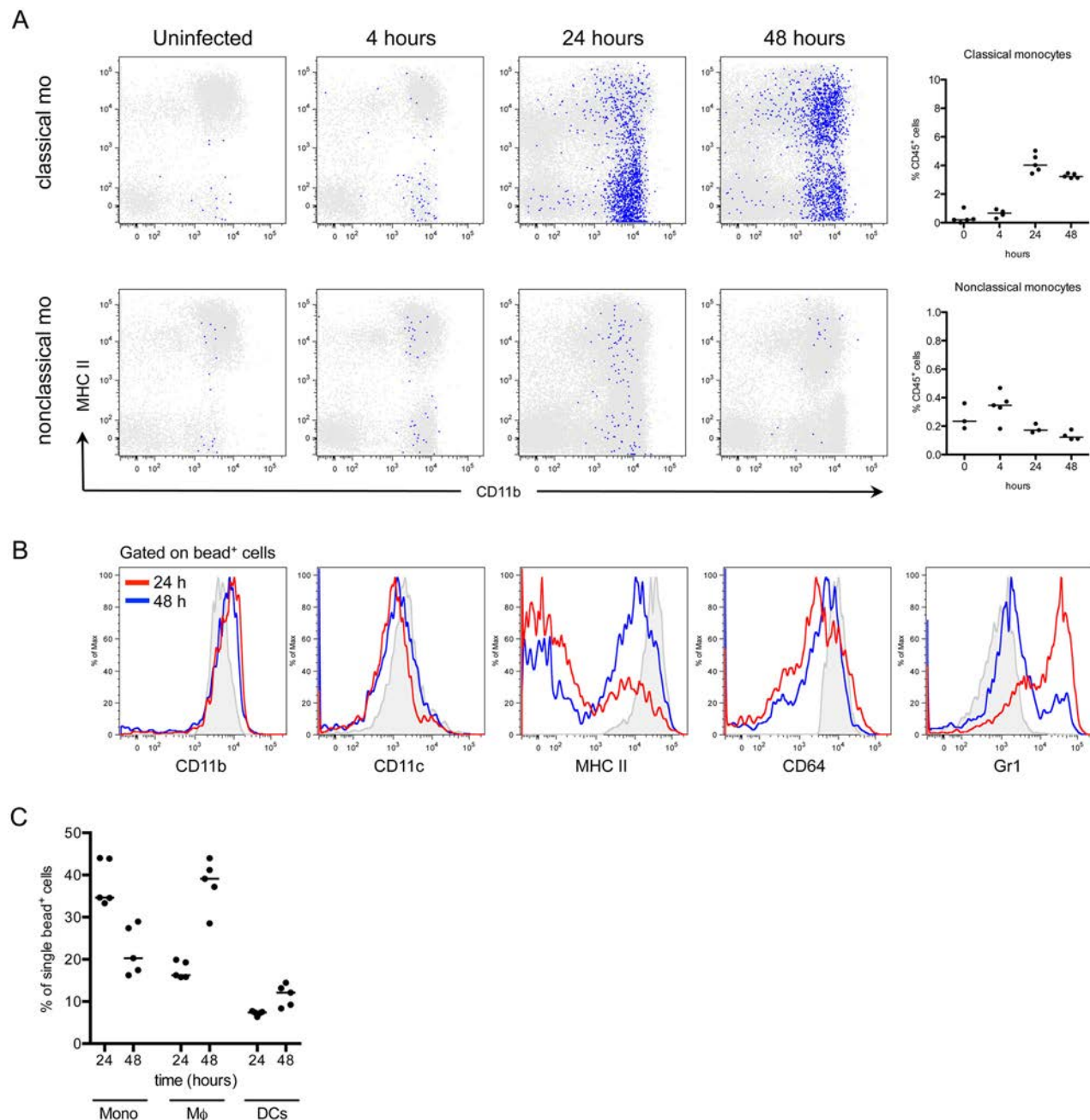
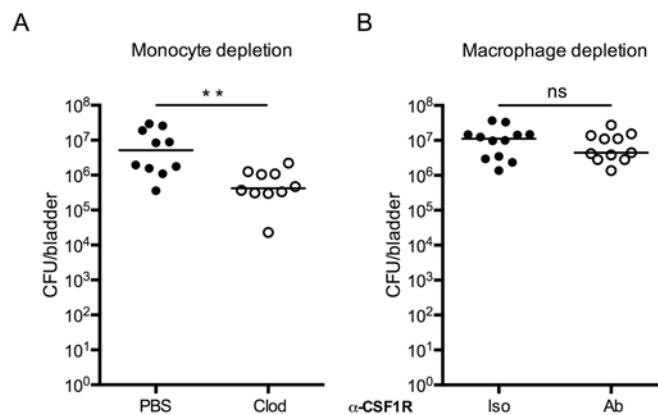


Fig 3. Classical monocytes robustly enter the bladder and become macrophages. In naïve C57Bl/6 mice, monocyte subsets were labeled *in vivo* as described in Materials and Methods. The designation “classical mo” and “nonclassical mo” indicates the monocyte subset labeled. Mice were infected with UT189 24 hours after monocyte labeling and sacrificed at 4, 24, and 48 hours P.I. for flow cytometry. (A) Representative cytometry plots are shown, where gated bead⁺ cells (blue) are overlaid on all CD45⁺ bladder cells (gray). Graphs depict the quantitation of infiltrating monocytes over time, note difference in y-axis. (B) Histograms show the bead⁺ cell phenotype in the bladder over time after classical monocyte labeling (red lines—24 hours P.I., blue lines—48 hours P.I., and gray histograms—resident macrophages at 4 hours P.I., for reference). (C) Graphs show the percentage of all bead⁺ CD45⁺ cells after classical monocyte labeling in infected bladders by immune cell subset at 24 and 48 hours P.I. Each dot is one mouse, lines are medians. In (A) and (B), single representative bladders are displayed. Experiments were performed 3 times with 3–5 mice per group.

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Primary infection



Challenge infection

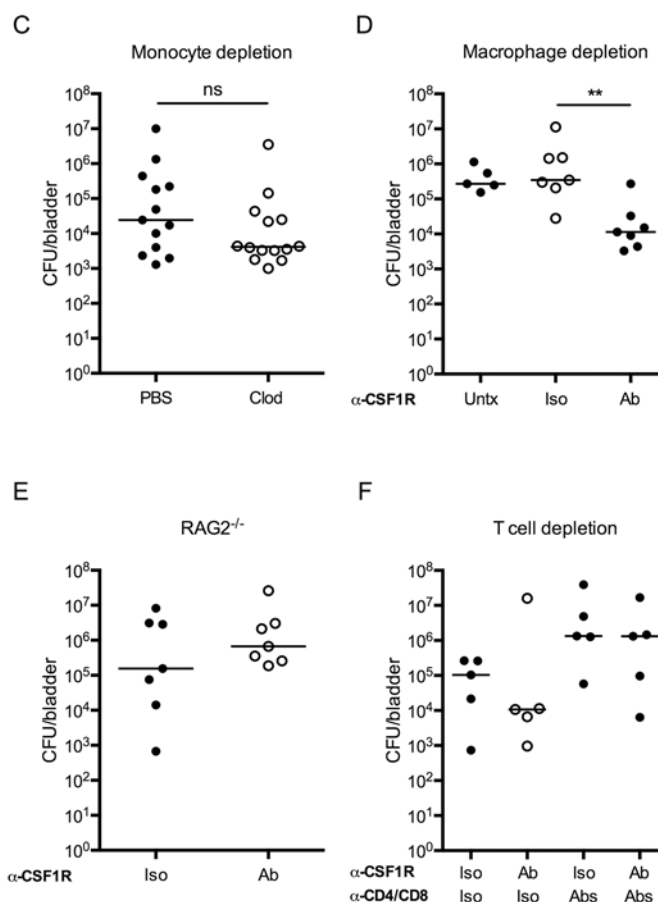


Fig 4. Macrophage depletion improves the adaptive response to UPEC infection. Graphs show CFU per bladder 24 hours post primary infection (A) in mice treated with PBS or clodronate liposomes (Clod) to deplete monocytes 15–18 hours prior to infection and (B) in mice treated 24 hours prior to primary infection with isotype control (Iso) or CSF1R antibody (Ab). (C–F) Mice were given a primary infection, allowed to resolve, and 3 to 4 weeks P.I., mice were challenged with an isogenic UPEC strain, as in Fig 1A. Graphs show CFU per bladder 24 hours post challenge infection in (C) wildtype mice treated with PBS or clodronate-loaded liposomes (Clod) to deplete monocytes 15–18 hours prior to primary infection, (D) isotype (Iso) or

CSF1R antibody (Ab) treated mice to deplete resident macrophages 24 hours prior to primary infection, or left untreated (Untx), (E) RAG2^{-/-} mice treated with isotype (Iso) or CSF1R antibody (Ab) prior to primary infection or, (F) wildtype mice treated with isotype (Iso) or CSF1R antibody (Ab) to deplete resident macrophages and isotype (Iso) or anti-CD4 and anti-CD8 antibodies (Abs) to deplete T cells, prior to primary infection. Each dot is one mouse, lines are medians. Experiments were performed 2–4 times with 4–7 mice per group. ***p* = 0.0021, Mann-Whitney; ns: not significant.

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that macrophage depletion at the time of primary infection positively impacts the capacity to generate a T cell-dependent adaptive immune response against UPEC.

Effector cell infiltration and cytokine secretion are unchanged after macrophage depletion during infection

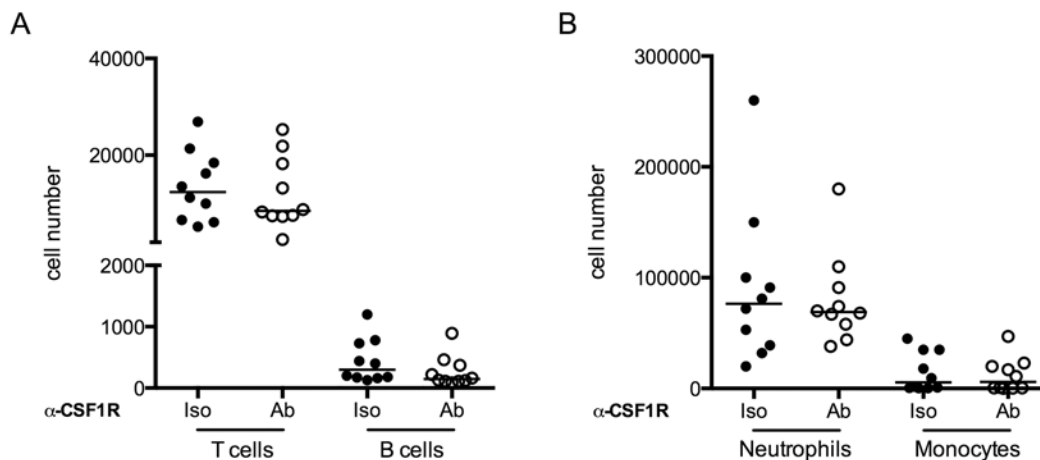
To investigate potential mechanisms mediating improved clearance after macrophage depletion in a challenge infection, we first focused on events following the challenge. We depleted macrophages and infected mice as above. When all mice had resolved the primary infection, we challenged the mice and 24 hours post-challenge, mice were sacrificed. We assessed immune cell infiltration into the bladder by flow cytometry and surprisingly, we did not observe differences in the number of T cells, B cells, neutrophils, or monocytes infiltrating the bladder after challenge infection ([Fig 5A and 5B](#)). These data suggest that improved bacterial clearance is not mediated by increased numbers of innate or effector cells. In addition to cell infiltration, we evaluated UPEC-specific IgA in the urine over time, however, the levels of UPEC-specific IgA were at the limit of detection of the assay, as has been reported in other studies [[7](#)].

Macrophages can influence the generation of an adaptive immune response through the modulation of the cytokine microenvironment [[37,38](#)]. Thus, we evaluated cytokine expression in the bladder 24 hours after a primary infection in control or antibody-treated mice by luminex technology. Notably, we did not observe significant differences between isotype-treated and antibody-depleted mice in any of the 32 cytokines evaluated ([Fig 5C](#) and [S6 Fig](#)), suggesting that cytokine expression is not significantly impacted at this timepoint in infection.

Macrophages are the predominant APC to acquire UPEC early after infection

Given that we found no differences in effector cell infiltration or cytokine expression, we considered whether macrophages sequester bacteria during infection. To test which immune cells acquire UPEC during infection, we utilized our kanamycin-resistant UTI89 strain, which also expresses MARS red fluorescent protein (UTI89-RFP) ([S7A and S7B Fig](#)). Importantly, when we infected mice with our ampicillin-resistant UTI89 strain expressing GFP, we could not clearly differentiate between UTI89-GFP-containing cells and the background autofluorescence of bladder macrophages ([S7A Fig](#)) or urothelial cells ([S2 Fig](#)). Importantly, UTI89-RFP had similar infectivity *in vitro* and *in vivo* compared to the parental UTI89 strain and UTI89-GFP ([S7C and S7D Fig](#)). Mice were instilled with 10⁷ CFU of UTI89-RFP and sacrificed at 4, 24, and 48 hours P.I. to analyze their bladders by flow cytometry. UTI89-RFP-containing cells were identified by gating CD45⁺ cells with RFP fluorescence levels greater than those in bladders infected with the non-fluorescent parental UTI89 strain ([Fig 6A](#)) and their phenotypes were determined based on expression of cell-specific markers as in [Fig 2](#). At 4 hours P.I., the majority of UTI89-RFP⁺ cells were in the MHC II⁺ cell compartment. At 24 and 48 hours P.I., UTI89-RFP⁺ cells were evenly distributed between the MHC II⁺ and II⁻ gates ([Fig 6B](#)). The MHC II⁻ cells containing bacteria at 24 and 48 hours were primarily CD11b⁺ Ly6G⁺ Ly6C⁺

Challenge infection



Primary infection

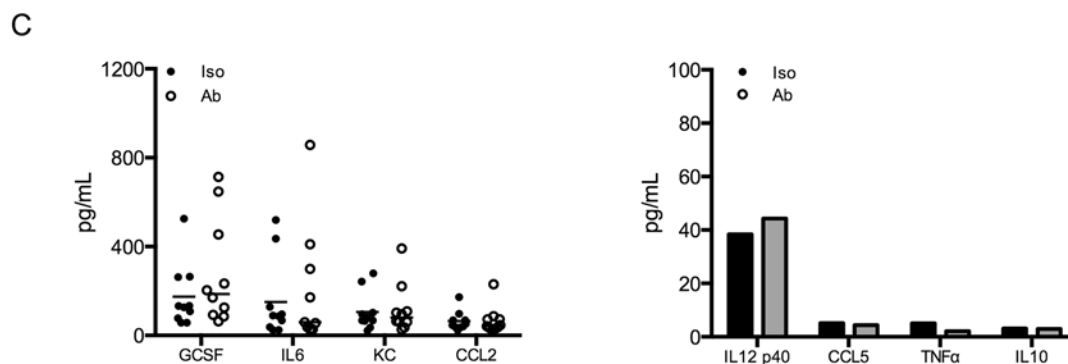


Fig 5. Macrophage depletion does not impact effector cell infiltration or cytokine expression. (A-B) Female C57Bl/6 mice were infected and then challenged with UPEC after resolution of their primary infection, as in Fig 1A. Twenty-four hours post-challenge, cellular infiltration into the bladder was evaluated by flow cytometry. Graphs depict cell number per bladder of the indicated populations. Experiment was repeated 3 times with 5–7 mice per group. (C) Mice were depleted with anti-CSF1R antibody and infected with 1×10^7 CFU of UT189 24 hours after depletion. Mice were sacrificed 24 hours P.I. and bladders were homogenized. Samples were stored at -80°C until all samples could be assessed together by Luminex multi-analyte profiling. Graphs depict the expression levels of selected cytokines in isotype antibody treated (black dots) and depleting-antibody treated (open circles) mice. Analytes are grouped by high expression (left graph) and low or no expression (right graph). Each dot represents a mouse, experiment performed 2 times with 5 mice per group and pooled, lines are medians. Additional analytes are shown in S6 Fig.

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neutrophils and $\text{CD11b}^+ \text{Ly6G}^- \text{Ly6C}^+$ monocytes. Among the $\text{UPEC}^+ \text{MHC II}^+$ cells, the majority exhibited a macrophage phenotype (Fig 6C). Notably, two subpopulations were distinguishable in the macrophage gate at 24 and 48 hours P.I., representing resident ($\text{CD64}^{\text{hi}} \text{F4/80}^{\text{hi}} \text{Ly6C}^-$) and monocyte-derived macrophages ($\text{CD64}^{\text{int}} \text{F4/80}^{\text{int}} \text{Ly6C}^+$) (Fig 6C). Indeed, while the number of DCs harboring UT189-RFP changed very little, the number of macrophages containing bacteria increased more than 7-fold at 24 hours and remained elevated at 48 hours (Fig 6D). At all timepoints analyzed, macrophages harbored approximately 60–80% of the bacteria found within the MHC II^+ APC compartment, demonstrating that macrophages were the primary cell type to phagocytose bacteria at early timepoints post-infection (Fig 6E).

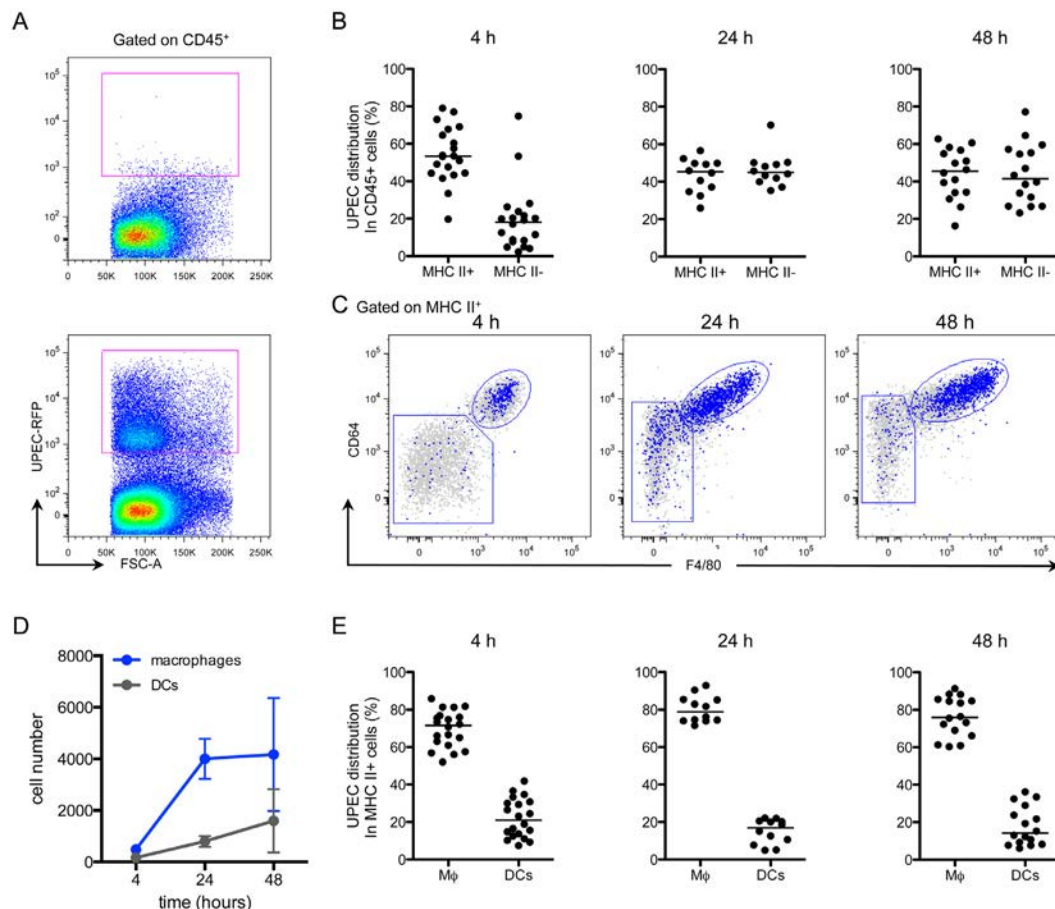


Fig 6. Macrophages preferentially take up UPEC at early times post-infection. Female C57Bl/6 mice were instilled with UT189-RFP and bladders were processed for flow cytometry at 4, 24, and 48 hours. (A) Representative plots, gated on CD45⁺ cells, depict the fluorescence level of UT189-RFP in bladders, as compared to bladders infected with the nonfluorescent parental UT189 strain. (B) Graphs depict the distribution of UPEC between MHC II⁺ and MHC II⁻ cell populations (gated first on CD45⁺ cells) over time. (C) Representative flow plots display the distribution of bacteria in the MHC II⁺ cell populations by overlay of bacteria⁺ cells (blue) on top of all CD45⁺ cells (gray). (D) Graph depicts the number of UPEC⁺ macrophages and DCs over time. (E) Graphs show the distribution of UPEC in CD45⁺ MHC II⁺ macrophages and DCs as a percentage of UPEC acquired by all MHC II⁺ cells. In (B) and (E), each dot represents one mouse, lines are medians. In (A) and (C), single representative bladders are depicted. Experiments were performed 4 times with 4–7 mice per group and results pooled in (B), (D), (E).

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Macrophage depletion leads to increased phagocytosis of UPEC by DCs

Macrophages can influence the generation of an adaptive immune response by antigen sequestration, as has been observed in the lung, hindering antigen presentation and subsequent T cell priming by DCs [39–42]. More specifically, it has been proposed that DC migration and the induction of adaptive immunity can only occur in the lung after the phagocytic capacity of macrophages has been saturated and excess antigen is available to DCs [42,43]. To test the hypothesis that antigen availability during primary infection impacted initiation of an adaptive response during UTI, we infected untreated mice with 10^7 , 10^8 , or 10^9 CFU of UPEC. When the animals had resolved the infection, they were challenged with 10^7 CFU of an isogenic UPEC strain and 24 hours post-challenge, sacrificed to determine bacterial burden. Notably, we did not observe a difference in the number of bacteria per bladder after a primary challenge despite increasing the inoculum 100-fold (S8A Fig). Furthermore, we did not observe any

differences in bacterial burden after challenge among the three groups (S8B Fig). Thus, increasing the number of bacteria during primary infection did not improve the generation of an adaptive immune response during UTI, however it is possible that we did not saturate bladder resident macrophages with bacteria.

To directly test whether macrophages were physically sequestering UPEC in the bladder during UTI, we evaluated bacteria uptake in the bladder in the context of macrophage depletion. Mice were depleted or not of macrophages by anti-CSF1R antibody, infected with UTI89-RFP and sacrificed 24 hours P.I. The distribution of UPEC was altered in mice depleted of macrophages as compared to the control group. More bacteria localized to MHC II⁺ cells (Fig 7A and 7B), potentially explaining why macrophage depletion did not impact bacterial clearance after primary infection. The percentage of neutrophils containing bacteria was specifically increased in macrophage-depleted mice, likely compensating for the lack of monocytes and resident macrophages (Fig 7B). In MHC II⁺ cell populations, while the total number of DCs in infected bladders was unchanged (Fig 7C), we observed a significantly greater percentage of DCs had taken up UPEC in macrophage-depleted animals (Fig 7D). Notably, the percentage of UPEC-containing resident and MHC II⁺ monocyte-derived macrophages was not different between the isotype and depleting antibody groups (Fig 7E).

Discussion

UTI is unusual in that it is a common infection that recurs with high frequency, particularly in otherwise healthy adult women [1], suggesting a defect exists in the ability to mount an adaptive immune response to UPEC. We observed that the absence of B and/or T cells or DCs impaired the host's capacity to clear bacteria after a challenge infection, confirming that adaptive immune responses are primed during UTI. Although this was an expected result, surprisingly, it has never been formally demonstrated in the literature until now. We further demonstrated that while immune responses are primed, they neither prevent reinfection nor eliminate the bacterial reservoir established during primary infection. To shed light on potential mechanisms preventing the development of effective adaptive immunity to UPEC infection, we focused on the role of MHC II⁺ cells as they are the key initiators of adaptive immunity. Unexpectedly, in the context of a challenge infection, resident macrophage depletion improved the host's ability to eliminate bacterial load. Importantly, macrophages were depleted prior to the first infection; however, they were present in normal numbers at the time of challenge infection. Notably, this improvement was dependent on the adaptive immune system as the phenotype was lost when macrophages were depleted in RAG2^{-/-} mice or in mice depleted of T cells. Given that macrophages were the principal APC to acquire UPEC early in infection, these data suggest that macrophages subvert initiation of a robust adaptive immune response during UTI.

To understand the mechanism of macrophage subversion of adaptive immunity during UTI, we evaluated events post-challenge and post-primary infection. We observed a significant infiltration of T cells and a smaller infiltration of B cells post-challenge but no major differences in cell numbers between the control and treated groups. At this time, we cannot rule out potential qualitative differences in the activation or specificity of the infiltrating effector cells, including T cells or possibly NK T cells, which may play a role in kidney infection [44]. As macrophages repopulated the bladder before challenge infection, we hypothesized that the impact on adaptive immunity occurred in the first few hours or days following primary infection. To explore the possibility of antigen sequestration, we evaluated which immune cell populations acquired UPEC in the bladder in the absence of macrophages. We observed an increase in the percentage of DCs containing bacteria. DCs are key players in initiating adaptive

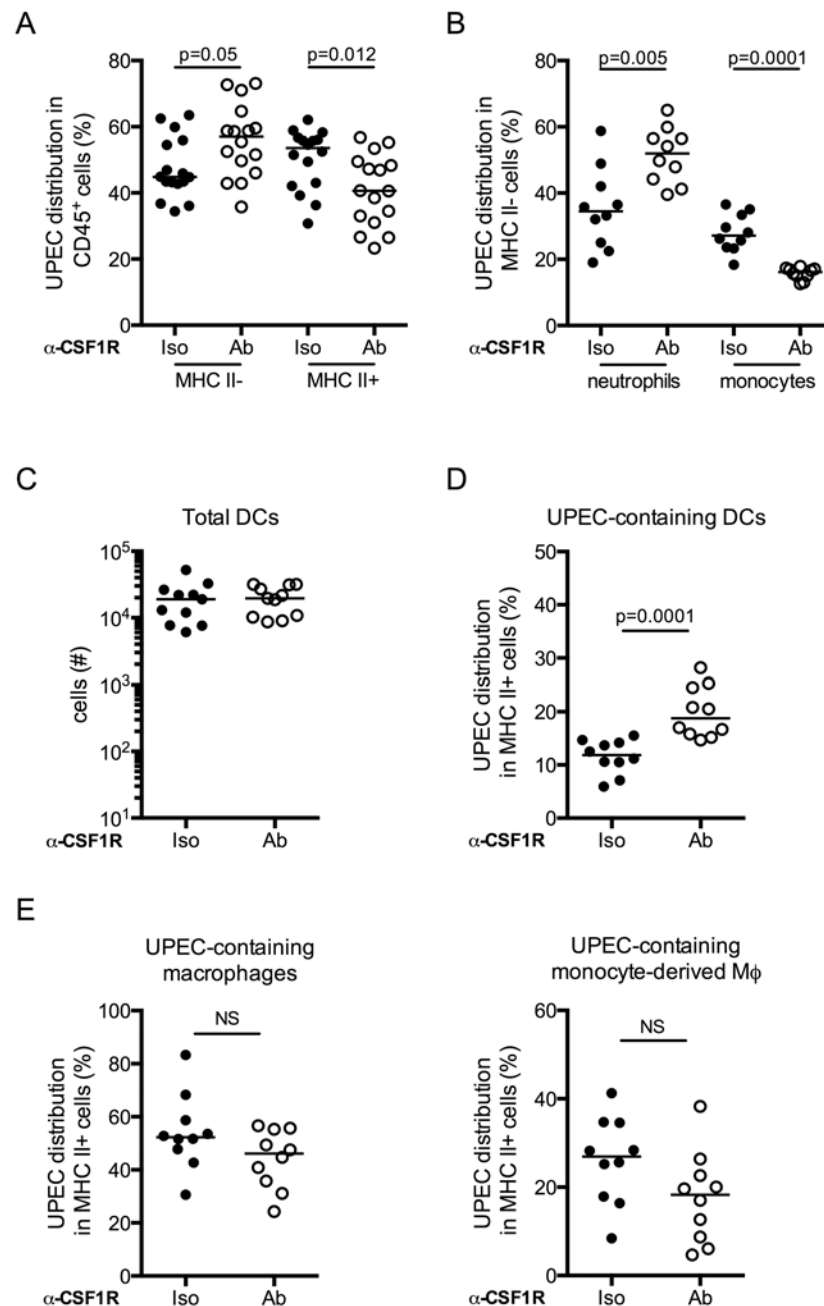


Fig 7. Dendritic cells acquire more bacteria in the absence of macrophages. Macrophages were depleted or not and mice were infected. Twenty-four hours post-primary infection, bladders were analyzed by flow cytometry to determine distribution of UPEC in (A) the MHC II⁻ and MHC II⁺ compartments, (B) neutrophils and monocytes. (C) Plot depicts the total number of DCs present in the bladder 24 hours post-primary infection. (D-E) Plots depict the percentage distribution of UPEC in the MHC II⁺ compartment by cell population (D) DCs and (E) resident macrophages and monocyte-derived macrophages. Each dot represents one mouse, lines are medians. Experiments were repeated 3 times with 4–7 mice per group in each experiment and results pooled. p-values indicated on graphs, Mann-Whitney test.

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immune responses, and we found that they can do so from the bladder mucosa in the context of UTI. Indeed, even a partial depletion of bladder-resident DCs, prior to primary infection, rendered animals less capable of clearing bacteria after challenge infection. The intermediate clearance phenotype observed in DT-treated mice may have been mediated by DCs remaining after depletion or by DCs repopulating the tissues before the primary infection was resolved, permitting delayed antigen presentation. Thus, our data suggest that the more efficacious adaptive immune response against UPEC observed during macrophage depletion may be mediated by the increase in the percentage and number of DCs carrying bacteria. Notably, however, the proportion of DCs containing UPEC in infected bladders was less than 5% of total DCs present in mice, suggesting that only a very small number of antigen-carrying DCs are required to mount an adaptive immune response during UTI.

Macrophages outnumbered DCs in both naïve and infected bladders and were the principal cell to acquire UPEC. Our findings contradict a recent study in which Schiwon *et al.* suggest that bladder-resident macrophages sense UPEC infection, but do not phagocytose bacteria [14]. As an explanation for this apparent discrepancy, we found that MHC II⁺ cells containing GFP-expressing bacteria were indistinguishable from autofluorescent but uninfected cells. We engineered UTI89 to express a red fluorescent protein to specifically overcome the challenge of distinguishing naturally autofluorescent cells in the bladder (*e.g.*, macrophages and urothelial cells) from those containing UPEC. Lending credence to this interpretation, the authors also did not detect GFP-expressing UPEC in urothelial cells [14], which are invaded during the course of UTI, as their autofluorescence also likely masked the GFP signal [45,46].

We evaluated macrophages because of their prominent role in bacterial acquisition. However, even when the majority of UPEC was captured by macrophages at early timepoints P.I., their depletion did not impact bacterial clearance after the primary infection. This apparent contradiction may be explained by the increased bacterial uptake by neutrophils observed in the absence of macrophages. Our data support a model in which macrophages sequester bacteria from DCs early in infection, however we cannot rule out that depletion of macrophages alters the microenvironment during infection, despite our negative findings in bladder homogenates. Indeed, a recent study suggests that IL-10 expression from mast cells suppresses adaptive immunity to UPEC [7]. However, in the course of our study, we found few mast cells in naïve bladder tissue. Furthermore, multi-analyte cytokine analysis revealed no striking differences between control and depleted mice and we could not detect IL-10 expression in this or a prior study [11]. The reasons for this are unclear, however may be due to the significant variation that exists in the genomes of commonly used strains such as cystitis strain UTI89, pyelonephritis strains J96, 563, CFT073, and clinical isolates (see phylogenetic tree in [47]).

Having defined an early role for resident macrophages and DCs during UTI, our work significantly advances the understanding of how adaptive responses to UPEC are achieved. However, we still do not completely understand how the adaptive immune system eliminates UPEC. Though we were not able to detect UPEC-specific antibodies above the limit of detection of our assay, others have identified that antibodies against the bacteria are generated during infection [17–19]. Thus, we hypothesize that the protection induced during UTI following a primary infection is mediated by an antibody response; however T cells may also play a critical role in the killing of infected cells. With respect to the role of the bladder macrophage, our data point to a barrier in the immune system that must be overcome, particularly for patients with recurrent UTI. Although macrophage sequestration of particulate antigen in the lung has been described, this is, to the best of our knowledge, the first study to propose a role for the physical sequestration of antigen during live bacterial infection. Strategies that increase DC number or migration may overcome the subversion imposed by macrophages, providing a viable solution to treat patients with recurrent UTI.

Materials and Methods

Ethics statement

At Mount Sinai School of Medicine, mouse experiments were conducted in accordance with approval of protocol number LA11-00003 by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine, which adheres to the guidelines put forth by the Animal Welfare Act and the Public Health Service policy on Humane Care and Use of Laboratory Animals. At Institut Pasteur, mouse experiments were conducted in accordance with approval of protocol number 2012–0024 by the Comité d'éthique en expérimentation animale Paris Centre et Sud (the ethics committee for animal experimentation), in application of the European Directive 2010/63 EU. In our experiments, mice were anesthetized either by inhalation of isoflurane (3–4%) or by injection of 100 mg/kg ketamine and 5 mg/kg xylazine and sacrificed either by cervical dislocation or carbon dioxide inhalation.

Bacterial strains

The human UPEC cystitis isolate, UTI89 (kind gift from Scott Hultgren) [46], and the fluorescent protein-expressing strains UTI89-RFP and UTI89-GFP were used for infection. Briefly, fluorescent bacteria were engineered using lambda red recombination [48] to introduce an *aphA-marsRFP* or *bla-GFP* cassette in the UTI89 chromosome at the *attB* lambda phage integration site. UTI89 is sensitive to antibiotics, UTI89-RFP is resistant to kanamycin, and UTI89-GFP is resistant to ampicillin. Bacteria were grown overnight in static cultures at 37°C in Luria-Bertani broth (LB) in the presence of antibiotics (kanamycin 50 µg/mL or ampicillin 100 µg/mL) where appropriate.

Cell lines and *in vitro* invasion assay

The mouse urothelial cell line NUC-1 [49] was used to evaluate the *in vitro* invasion efficacy of each UTI89 strain. Fifty thousand cells were infected with UTI89, UTI89-GFP, or UTI89-RFP at increasing MOIs. Thirty minutes post-infection, cells were washed, lysed, and serial dilutions were plated. Percent invasion was calculated by dividing the number of bacteria inside the cells by the inoculum x 100.

Mice and infections

Female C57BL/6 mice between 6 and 8 weeks old were from The Jackson Laboratory or Charles River. CD11c-DTR mice were a kind gift from Marc Lecuit and Claude LeClerc (Institut Pasteur). RAG2^{-/-} mice were a kind gift from Antonio Freitas (Institut Pasteur). Briefly, mice anesthetized with isoflurane (4%) or 100 mg/kg ketamine and 5 mg/kg xylazine were infected with 10⁷ colony-forming units (CFU) of one of two UTI89 strains in 50 µL PBS via a catheter introduced into the urethra [20] except in the inoculum escalating experiment, where mice received 10⁷, 10⁸ or 10⁹ CFU in 50 µL PBS. To calculate CFU, bladders were aseptically removed and homogenized in 1 mL PBS. Serial dilutions were plated on LB agar, with or without antibiotics, as required. For challenge infection experiments, mice were infected with one of the two fluorescent strains of UTI89, expressing antibiotic resistance (kanamycin or ampicillin) (See Fig 1A). Once the primary infection cleared, 3 to 4 weeks, mice were infected with 10⁷ CFU of an isogenic UTI89 strain with a different antibiotic resistance. The strain used for the challenge infection was determined by that used in the primary infection, such that the antibiotic resistances were different between the primary and challenge infection, *e.g.*, UTI89-GFP for the primary and UTI89-RFP for the challenge infection. Importantly, both strains were used as the

primary or the challenge strain in different experiments. Resolution of infection was monitored by plating urine every 5–6 days on antibiotic-containing plates.

Irradiation, bone marrow cell transfer, diphtheria toxin treatment

C57Bl/6 mice were irradiated with a single dose of 5–6 gray in an x-ray irradiator at 6 weeks of age. Animals were reconstituted with $1.6\text{--}3.2 \times 10^6$ total bone marrow cells from CD11c-DTR mice 6 hours after irradiation. Mice were allowed to reconstitute for a minimum of 12 weeks and reconstitution was evaluated by flow cytometry of congenic markers. 24 and 48 hours prior to infection, mice were administered 4 ng/g of diphtheria toxin I.V. Depletion efficiency was tested in each batch of chimeric mice prior to experimentation.

Flow cytometry of bladder tissue

At indicated timepoints, bladders were removed and minced with dissection scissors into tubes containing digestion buffer kept at 4°C. Minced tissue was then incubated at 37°C in 1 mL of digestion buffer containing 0.34 U/mL of Liberase TM (Roche) and 100 µg/mL of DNase in PBS. Tubes were vigorously shaken by hand every 15 minutes. 45 minutes to one hour post-incubation, when the tissue had a glassy, transparent appearance and was almost entirely digested, digestion was stopped by adding several mL of PBS supplemented with 2% FBS and 0.2 µM EDTA. The entire bladder digest was passed through a 100 µm cell strainer to obtain a single cell suspension. Gentle pressure was applied to any tissue remaining in the strainer. Samples were washed, Fc receptors blocked, and stained with antibodies listed in [Table 2](#). Total cell counts in the bladder were determined by the addition of AccuCheck Counting beads (Invitrogen) to a known volume of sample after staining, just prior to cytometer acquisition. Gating strategies for all cell populations except for neutrophils are depicted in [Fig 2](#). Neutrophils were identified as MHC II⁺, CD11b⁺, Ly6G⁺, Ly6C⁺, SiglecF⁺, and F4/80⁺.

Flow cytometry of blood

To identify cell populations in the circulation, whole blood was incubated with BD PharmLyse, (BD Bioscience) and subsequently stained with antibodies indicated in the [Table 2](#). Samples were acquired on a BD LSRFortessa using DIVA software and data were analyzed by FlowJo (Treestar) software. Total cell counts in the blood were determined by the addition of Accu-Check Counting beads (Invitrogen) to 10 µL of whole blood diluted in 1-step Fix/Lyse Solution (eBioscience).

Monocyte bead labeling

In vivo bead labeling of classical and nonclassical monocytes was performed as previously described [32]. Briefly, classical monocytes were labeled by I.V. administration of 200 µL clodronate liposomes to transiently deplete all monocytes and then by I.V. injection of 1 µM non-degradable fluorescent particles 24 hours later. Nonclassical monocytes were labeled by injection of 1 µM nondegradable fluorescent particles without prior monocyte depletion. Labeling efficiency was confirmed by flow cytometry.

Immune cell depletion

To deplete monocytes, wildtype C57BL/6 mice received I.V. injection of 200 µL of clodronate liposomes (or PBS control liposomes) 15–18 hours prior to infection [35]. Anti-CSF1R antibody (2 mg/mL, clone AFS98, eBioscience) was used to deplete bladder-resident macrophages. Animals received two I.V. injections, on consecutive days, of anti-CSF1R antibody or isotype

control (clone eBR2a, eBioscience). We administered 400 $\mu\text{g}/\text{mouse}$ on day 1 and 200 $\mu\text{g}/\text{mouse}$ on day 2, to decrease the impact on circulating monocytes. To deplete T cells, 100 μg of CD4 (clone GK1.5, Bio X Cell) and 100 μg of CD8 (clone YTS 169.4, Bio X Cell) per mouse were injected together intraperitoneally 24 hours prior to primary infection. 200 μg of isotype control (clone LTF-2, Bio X Cell) per mouse was injected intraperitoneally. The depletion was repeated 5 days post-infection, and once a week to maintain the depletion until challenge infection.

Luminex MAP analysis

Mice were infected with UTI89 and bladders removed 24 hours P.I. Bladders were homogenized with a handheld tissue grinder in 1 mL PBS on ice. After removal of a 100 μL aliquot to calculate CFU by serial dilution, bladder homogenates were clarified by microcentrifugation (13K, 4, 5 minutes) and stored at -80°C until assessment by Luminex Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Premixed 32-Plex, according to the manufacturer's recommendations (Merck Millipore) [11]. All samples were assessed together to avoid inter-assay variability. Just prior to analysis, after thawing, samples were centrifuged a second time to remove any cell debris.

Statistical analysis

GraphPad Prism was used to evaluate statistical significant. Graphs depict medians and statistical significance was determined by the nonparametric Mann-Whitney test.

Supporting Information

S1 Fig. DT-mediated DC ablation. Irradiated C57Bl/6 mice were reconstituted with bone marrow from CD11c-DTR animals and allowed to rest for 12 weeks. Prior to infection, mice were treated two times with PBS (-) or 4 ng/g diphtheria toxin (+). 24 hours post-treatment, a cohort of animals were analyzed by flow cytometry to assess the extent of depletion in the bladder. Graph depicts the number of DCs in the bladder in PBS or DT treated mice. Each dot is one mouse, lines are medians. Depletion efficiency was tested in each batch of chimeric mice prior to experimentation, $n = 2-4$ mice per group.
(TIF)

S2 Fig. Bladder autofluorescence. (A) Gating strategy for whole bladder digests. Bladders from naïve mice were processed as described in Materials and Methods. The entire bladder preparation was acquired and live cells were gated based on their forward and side scatter properties (top left). CD45^{+} cells were identified (top, middle), however, this population contained a large contaminating cell population (gated in pink, top right), particularly when CD45 was conjugated to fluorophores that emit near the emission wavelength of GFP. To eliminate the contaminating autofluorescent cells from our analyses, we selected single cells (FSC-W, SSC-W) versus MHC II staining (bottom, left, middle). The autofluorescent population was reduced by this strategy while immune cell populations remained (bottom, right) (B) Micrograph of the luminal surface of an *en face* whole mount prepared bladder stained only with DAPI (blue) to reveal DNA, to illustrate the intrinsic autofluorescence in this tissue. (C) Graphs depict the percentage decrease in the contaminating cell population (left) and the relative change in the myeloid cell populations in the bladder after each gating step (right, CD11b^{+} cells are derived from black gates and CD11c^{+} cells are derived from blue gates in (A), and contaminating cells are gated in pink).
(TIF)

S3 Fig. Immune cell ablation. (A-B) Mice were treated with PBS or clodronate liposomes (Clod) I.V. and 15–18 hours later, blood and bladder samples were obtained to evaluate immune cell depletion. Graphs depict the percentage of (A) monocytes and neutrophils in blood and (B) monocytes, macrophages, and DCs in the bladder after treatment. (C-D) Mice received two injections of anti-CSF1R antibody (Ab) or control isotype antibody (Iso) and 24 hours post-treatment, naive bladders were isolated to evaluate immune cell depletion. Graphs show the (C) percentage and cell number of macrophages and DCs in the bladder and (D) percentage of monocytes and neutrophils in the blood. (E) Mice were depleted of macrophages as in (C-D), however, bladders were evaluated for repopulation by macrophages 4 weeks after depletion, prior to challenge infection in additional cohorts of treated mice. Each dot represents one mouse. Experiments were repeated 2–4 times with 2–7 mice per group.

(TIF)

S4 Fig. CCR2^{-/-} mice are not impaired in bacterial clearance after primary infection. Graph depicts the CFU/bladder 24 hours post-primary infection in wildtype (WT) or CCR2^{-/-} mice. Experiment was repeated 2 times with 4–5 mice per group.

(TIFF)

S5 Fig. UPEC reservoirs are not altered in monocyte or macrophage depleted mice. Graphs depict CFU/bladder arising from the primary infecting strain in an experiment in which (A) monocytes or (B) macrophages were depleted prior to primary infection and then challenged with an isogenic strain and sacrificed 24 hours post-challenge. Each dot represents one mouse. Experiments were repeated 2–4 times with 2–7 mice per group.

(TIFF)

S6 Fig. Macrophage depletion does not impact cytokine expression post-primary infection. Mice were depleted with anti-CSF1R antibody and infected with 1×10^7 CFU of UTI89 24 hours after depletion. Mice were sacrificed 24 hours P.I. and bladders were homogenized. Samples were stored at -80°C until all samples could be assessed together by Luminex multi-analyte profiling, to avoid inter-assay variability. Graphs depict the expression levels of selected cytokines in isotype antibody treated (black dots, red medians) and depleting-antibody treated (open circles, blue medians) mice. Analytes are grouped by high expression (top) to low or no expression (bottom). Each dot represents a mouse, experiment performed 2 times with 5 mice per group and all data pooled.

(TIFF)

S7 Fig. Fluorescent UPEC strains. (A) Cytometry plots, gated on all CD45⁺ cells, depict GFP fluorescence (gated in pink with percentages) in mice either uninfected or infected with UTI89-GFP at 4 hours post-infection. (B) Fluorescence of UTI89-GFP and UTI89-marsRFP was confirmed by microscopy. (C) The mouse urothelial cell line, NUC-1, was infected with the parental UTI89, UTI89-GFP, or UTI89-RFP at an MOI of 1, 10, or 100. Cells were lysed and bacterial titers determined by serial dilution 30 minutes P.I. The percentage of invasion refers to the number of bacteria obtained after infection \times 100/number of bacteria in the inoculum. (D) Mice were instilled with 1×10^7 CFU of UTI89, UTI89-GFP, or UTI89-RFP. CFU per bladder were determined by serial dilution at 24 h P.I. Each dot represents one mouse. Experiments were repeated 2 times.

(TIF)

S8 Fig. Increasing bacterial inoculum in a primary infection does not improve the response to challenge infection. Female C57Bl/6 mice were instilled with 1×10^7 , 1×10^8 , or 1×10^9 CFU of UPEC and challenged with 1×10^7 CFU after resolution of the primary infection as in [Fig 1A](#).

(A) Plot depicts CFU 24 hours post-primary infection. (B) Graph shows CFU 24 hours post-challenge. Each dot represents one mouse. Experiments were repeated 2 times with 5–7 mice per group.

(TIFF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: GMB AMP GJR MLA MAI. Performed the experiments: GMB AMP MAI. Analyzed the data: GMB AMP GJR MLA MAI. Contributed reagents/materials/analysis tools: NvR. Wrote the paper: GMB AMP GJR MLA MAI.

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